Food Research

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INSIDE SCIENCE

THE VITAL STORY OF

Breakfast Cereals

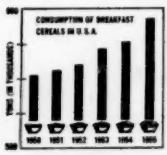
with essential vitamins and minerals restored

by Science Writer

New Edition

AMERICA LIKES BREAKFAST FOODS

Let no one doubt the popularity of breakfast cereals among Americans. The chart below traces the consumption of these fine foods between 1950 and 1955. During that period annual consumption rose by *76,000 tons*. In just one year, 1955, Americans ate 2½ lbs. of hot and 4.8 lbs. of cold cereals per person!



Why are breakfast cereals so well-liked? They are tasty; they are easily served; they appeal to busy homemakers, as well as institutional dietitians, because they are readily available in a variety of flavors at a modest cost. They add interest and value to an important but sometimes neglected meal —breakfast. Their use is extending to between-meal and party snacks, too.

Many grains are processed to make breakfast cereals: wheat, corn, oats, rice. Eaten with fruit and milk or light cream, they contribute an excellent combination of basic, flavorful, nutritious foods to the diet.

Better Foods for Better Health Through Restoration

The science of nutrition has advanced rapidly. In the manufacturing process of some cereals, some of the essential "B" vitamins and minerals are subject to some loss, just as with other foods. These losses are inescapable when such grains are prepared for human use. When this became known, manufacturers acted to overcome the losses. They adopted restoration.

Restoration simply means that certain important vitamins and minerals are restored to the cereal food during processing, so that the vitamin and mineral values in the finished product are generally equal to the whole grain values of those elements. Wheat, corn and rice products are customarily so treated. Vitamins B_1 (thiamine), B_2 (riboflavin), niacin (another "B" vitamin), and the mineral, iron, are those most widely restored. Vitamins C and D are also sometimes added.

Pre-sweetened cold cereals emphasize the nutritional importance of added vitamins. Increased calories require more "B" vitamins for best utilization of the food.

Why the Vitamins are Important

Physicians and diet experts have proved that vitamins are essential to prevent certain deficiency diseases and to contribute to robust good health.

Vitamin B_1 (thiamine) helps build and maintain physical and mental health. It is essential for normal appetite, intestinal activity, and sound nerves. A lack of this vitamin leads to beriberi, a rarity in the U. S. A., but still a very serious health problem in other parts of the world.

Vitamin B_2 (riboflavin) is essential for growth. It helps to keep body tissues healthy and to maintain proper function of the eyes.

Niacin is needed for healthy body tissues. Its use in the American diet has been largely responsible for the virtual disappearance of pellagra. a serious disease.

Vitamin D helps children develop normal teeth and bones. It prevents the development of certain abnormal bone conditions in adults.

Iron is essential for making good red blood and for the prevention of nutritional anemia.

Where Do the Vitamins Come From?

At about the same time that processing losses in breakfast cereals became known, other developments in the scientific world made available ample supplies of vitamins at economical prices. Thus, the nutritional contribution of some breakfast cereals could be, and was, greatly improved through restoration.

Since the early days of breakfast food restoration and of white flour and white bread enrichment, the world-famous firm of Hoffmann-La Roche has supplied top quality vitamins *by the tons*. Pioneering work in its laboratories and by its collaborators resulted in the "duplication" of some of nature's extremely complex substances. First, the chemical composition of the vitamin was learned. Second, the pure substance was isolated. Third, the "duplicate" was made by synthesis. And fourth, the laboratory techniques were extended to large scale commercial operations.

The manufactured "duplicate" is identical chemically and in biological activity with nature's own product. A vitamin is still a vitamin regardless of whether nature or man made it. So efficient is large-scale manufacturing, that vitamins are sold at a lower cost than if they were extracted from natural sources.

This article is one of a series devoted to the story of vitamin enriched or restored cereal products: white flour, white bread and rolls, corn meal and grits, macaroni products, white rice, breakfast cereals, farina. Reprints of this article, of any other in the series, or of all are available without charge. Please send your request to the Vitamin Division, Hoffmann-La Roche Inc., Nutley 10, New Jersey. In Canada, Hoffmann-La Roche Ltd., 286 St. Paul Street, West; Montreal, Quebec.

EFFECT OF SALINE AND EGG SHELL MEMBRANE ON BACTERIAL GROWTH

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(Manuscript received June 27, 1956)

Bacterial penetration of the hen's egg is thought to be influenced by such natural barriers as the shell mucin, shell, and shell membranes. Numerous studies of egg infection indicate a general agreement among workers that the shell and mucin coating play a simple mechanical role in protecting the egg against microbial invasion *(4, 5, 10, 13, 19)*. With a few exceptions, infection studies heretofore have failed to reveal any direct evidence that membrane penetration is accomplished by other than physical means, i.e., through the pores. On the other hand, Stokes and Osborne *(15)* suggested the possibility that enzyme activity is involved in membrane penetration. It has been frequently indicated that the shell membrane is one of the egg's defensive mechanisms against bacterial invasion *(4, 10, 11, 12, 13, 19)*. Stuart and McNalley *(16)* demonstrated that, when eggs were coated with a culture of *Pseudomonas aeruginosa* and examined three hours later, a greater number had infected membranes and albumen than eggs similarly treated and stored for 192 hours at 23° to 28° C. They explained their observation by showing that in a saline suspension of ground albumen-free membrane 80% of the inoculated cells were killed within four hours. Gillespie and Scott *(5)*, however, pointed out that this did not prove the existence of limited bactericidal properties in the membrane because no saline controls were used and the possibility of bacteria adhering to membrane particles was not eliminated. These workers also pointed out various indications that the outer membrane was not as easily penetrated as was once believed. Zagaevsky and Lutikova *(19)* observed that *Pseudomonas aeruginosa* and *Sarcina lutea* failed to penetrate into the white of eggs until the 15th to 20th day after infection. Romanoff *(13)* suggested that this failure was due to membrane resistance. However, more recent work in this laboratory has indicated that penetration can occur almost immediately under experimental conditions. Stokes and Osborne *(15)* have shown that under proper conditions of temperature and humidity non-motile and motile species of *Salmonella* are equally capable of effecting entry into the albumen. Miller and Crawford *(12)* observed that the shell membrane *in vitro*, when washed free of albumen, delayed multiplication of certain egg-spoilage bacteria. Stokes and Osborne *(14)* inoculated saline suspensions of ground washed membrane with *Salmonella* and *Pseudomonas* organisms and observed reduction in numbers of bacteria within one and five hours, followed by rapid increases after 22 hours incubation. The reduction rate was much more rapid in saline alone than Stuart and McNalley *(16)* reported, which

suggests substantial strain and species differences in sensitivity to diluent and possibly to membrane. Stokes and Osborne *(14)* attributed the apparent bactericidal effects of such suspensions to the toxic action of the saline, and the extensive growth after incubation to nutrients supplied by the membrane.

The influence of physiological saline on the viability of bacteria has long been a source of argument. If results reported by Stokes and Osborne *(14)* indicate "toxicity" of normal saline against a species of *Pseudomonas*, the desirability of this diluent is questionable as a suspending medium for bacterial plate-counting techniques. The literature reveals the highly controversial nature of this problem, although results disproving any seriously adverse effects of saline on viability of bacterial populations are in the majority *(1, 2, 3, 6, 7, 8, 9, 17, 18)*. It appeared desirable, therefore, to determine whether Ringer's Salt Solution, used routinely in this laboratory as a diluent, had any adverse effects on various *Pseudomonas* species. It seemed desirable also to test for bacteriostatic properties in the egg-shell membrane using a suspending medium in which relatively constant numbers of bacteria could be maintained over the test period. Such a medium was found, and egg-shell membrane was tested against two organisms differing widely in their susceptibility to inhibitory substances in the egg. Data are also presented on the effect of salt concentration on the growth of a *Pseudomonas* species.

MATERIALS AND METHODS

Determination of a satisfactory diluent. The diluents tested were:

Saline, 0.85%, buffered and unbuffered,
Distilled water, buffered and unbuffered,
Tap water, buffered and unbuffered,
Ringer's Salt Solution, buffered and unbuffered,
Sorensen phosphate buffer, M/15, pH 7.3.

Where indicated, diluents were buffered to pH 7.3 with M/15 Na_2HPO_4. The test organism consisted of a strain of *Pseudomonas*, resembled *Pseudomonas fluorescens* in biochemical characteristics, and was capable of producing typical "green-rot" spoilage of eggs. This strain had been freshly isolated from poultry-house litter. Cells from a 24-hour slant growth in Trypticase Soy Agar (BBL) were washed and centrifuged 3 times and resuspended in a small amount of sterile saline. This suspension gave an approximate turbidity of 12.3 expressed as per cent transmittance on a Model 310 Coleman Spectrophotometer and was comparable to that of the No. 10 tube of a McFarland Nephelometer. This represented a bacterial concentration of approximately 3×10^9 cells per ml. Test diluents were inoculated in duplicate with high and low numbers of organisms, representing final concentrations of approximately 6×10^8 and 3×10^4 cells per ml. respectively. Aliquots of each inoculated sample were further diluted and 1.0 ml. portions of the final dilutions were plated in triplicate immediately, at hourly intervals through 6 hours, and at 24 hours. Veal Infusion Agar (Difco) was employed as the plating medium. All plates were incubated at 27° to 30° C. for 72 hours prior to counting. Samples were held in a constant temperature water-bath at 30° C. between sampling periods. Aseptic methods were employed throughout. Bacterial numbers represent the average of each set of triplicate plates for each plating period and are expressed as per cent survivors.

Determination of optimum molar concentration for growth. In the second phase of this work, studies were made on the influence of various molar concentrations of NaCl on the growth of the test organism in order to ascertain the limits within which growth could be maintained at a stationary level and to determine the relative position of

physiological saline therein. The methods employed were essentially those of Hotchkiss *(9)*. A 4.0 M solution of Reagent Grade (A.C.S.) NaCl in 1.0% Bacto-Peptone, pH 7.3, was prepared, and, by two-fold serial dilution through 11 tubes, gradient concentrations ranging from 0.0075 M to 4.0 M were obtained. A control tube contained peptone solution only. One per cent peptone was employed as the basal medium since it supports growth satisfactorily; at this concentration the influence of the inorganic material in dried peptone—0.03 g. per 100 ml.—on growth of the test organism is negligible. Thirty-six tubes prepared in triplicate series of 12, with a final volume of 10.0 ml. per tube were each inoculated with 0.1 ml. of a 1:10 dilution (in sterile distilled water) of a 24-hour growth of the test organism in 1.0% Peptone, pH 7.3, and incubated 24 hours at 27° to 30° C. Determinations of pH on each tube of one series were made prior to and after incubation. Triplicate plate-counts were performed after incubation. The entire experiment was repeated three times, and the final results were expressed as averages.

Determination of the effect of membrane on growth. The inoculum consisted of a 24-hour Trypticase Soy Broth (BBL) culture of the test organism diluted with buffered saline (pH 7.3) to obtain a cell concentration of approximately $2x10^6$ cells per ml. Turbidity, measured as per cent transmittance on a Model 310 Coleman Spectrophotometer at a wave length of 650 mμ, was employed as a criterion of cell concentration.

Shell membranes were removed aseptically from 4 large (24 oz./doz.) White Leghorn eggs, weighed (wet weight) in a closed sterile container, suspended in 100 ml. sterile buffered 0.85% NaCl solution (pH 7.3), and homogenized in a VirTis high speed micro-homogenizer at 40,000 r.p.m. until a coarse dispersion was effected in which membrane particle size was no longer macroscopically discrete. A second sample of pooled membranes was prepared in the same manner but was washed to remove residual albumen by vigorous agitation through several (10 to 12) changes of buffered saline (pH 7.3). The membranes were considered albumen-free when no apparent turbidity or foaming occurred in the final wash solution. The homogenates were allowed to stand in suspension, with occasional agitation, under refrigeration (4° to 5° C.) for 3 days prior to use. It was considered that, by allowing the suspension to stand, maximum leaching of the membrane components by the suspending medium would occur. This observation was confirmed in a separate study in which the growth curve of the test organism in such a suspension was compared to that in a membrane suspension which was inoculated and used immediately after homogenization. The growth curves in the latter and in a saline control suspension were identical in that, unlike the growth pattern of the organism in the membrane suspension that had been allowed to stand prior to inoculation, they indicated a progressive decrease in viable cell numbers over an 8-hour incubation period. The third preparation consisted of 100 ml. of a buffered saline suspension of homogenized egg albumen (equivalent by weight to that of the membrane samples). The control was 100 ml. of 0.85%NaCl solution buffered to pH 7.3 with M/15 Na_2HPO_4. The suspensions and control were each inoculated with 1.0 ml. of the bacterial suspension, and 1.0 ml. aliquots plated in triplicate in Veal Infusion Agar (Difco) immediately, at hourly intervals through 6 hours, and at the end of a 24-hour period. All plates were incubated at 27° to 30° C. for 72 hours prior to counting. The test materials were incubated in a constant temperature water-bath at 30° C. between sampling periods. The conventional pour-plate method for enumerating bacteria was employed.

RESULTS AND DISCUSSION

The effect of various suspending media on survival of a green fluorescent pigment-producing strain of *Pseudomonas* is presented in Figures 1 and 2. Substantial decreases in numbers of organisms occurred in all solutions tested. In no case, however, were the precipitous drops observed as reported by Stokes and Osborne *(14)*. This does not necessarily indicate a conflict in results since the organisms used were different and the levels of inoculum were not the same. Buffering the diluents to pH 7.3 appeared to reduce the rate of bacterial destruction as compared to the respective

unbuffered solutions. This observation appears to be consistent with that of Holm and Sherman *(8)* who point out that although the effect of salts on growth is analogous to its effect upon chemical reactions, coagulation, and permeability, these effects are modified by the H-ion concentration of the medium. The slight difference between the growth effect of buffered and that of unbuffered saline on the test organism is probably not significant statistically. From these results it was decided to use 0.85% NaCl solution, buffered to pH 7.3 with M/15 Na_2HPO_4, as the suspending medium for the egg-shell membrane studies.

The buffered saline and buffered Ringer's Salt Solution produced extremely high numbers of bacteria following the initial period of reduction. No satisfactory explanation can be offered for this observation, although it is noteworthy that certain strains of *Pseudomonas* species are capable of multiplying and producing characteristic fluorescent pigment in an aqueous medium consisting of asparagine as the sole source of nitrogen and minute amounts of inorganic phosphate and sulfate ions. In the Reagent Grade NaCl used and in the distilled water there may

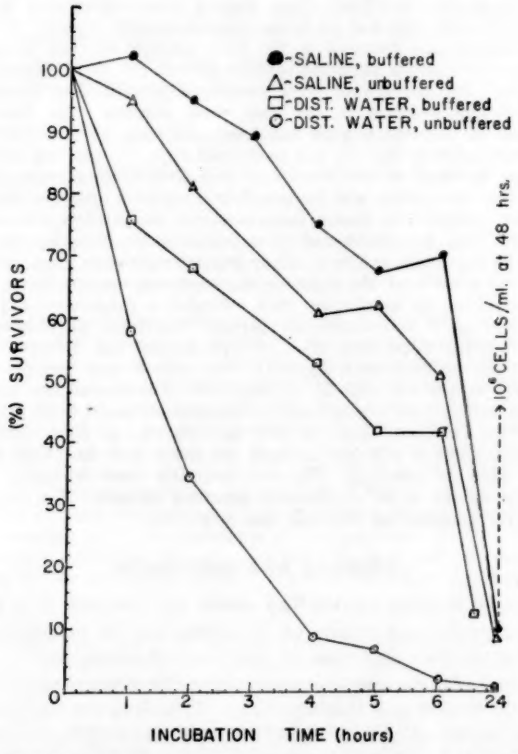

Figure 1. Survival curves of a "green-rot" producing species of Pseudomonas (PP-H) in various suspending media.

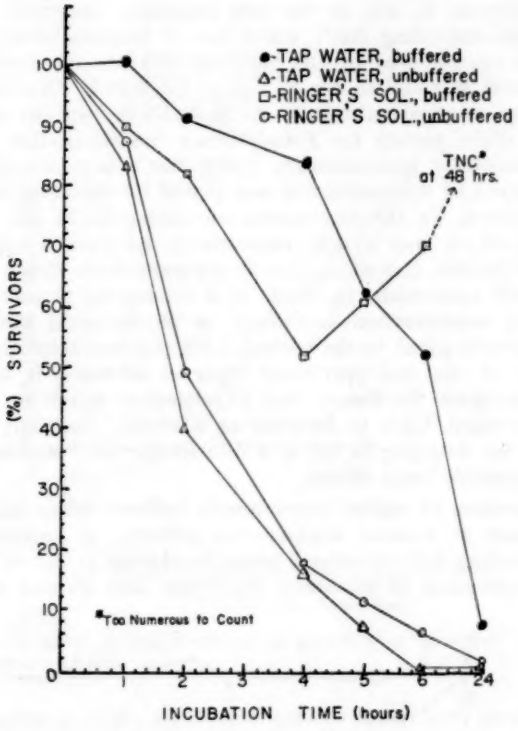

Figure 2. Survival curves of a "green-rot" producing species of Pseudomonas (PP-H) in various suspending media.

exist subminimal levels of these inorganic ions. Admittedly, these levels may not be sufficient to support growth over any extended period of incubation, but it is not inconceivable that they could retard the death rate of these organisms over a reasonable period of time and thus check the sudden drop in the survival curve that might obtain when distilled water alone is used as a suspending medium. Moreover, cultural and microscopical observations of the organisms present in the buffered saline diluent after 6 hours incubation differed morphologically and cytochemically from the parent cells, and were able to grow in nutrient media containing much higher NaCl concentrations. These findings suggest that salt-resistant variants were produced which could multiply in buffered saline and buffered Ringer's solution, presumably using parent cell debris as a source of nutrients.

Figure 3 demonstrates the effect of various molar concentrations of NaCl upon growth of the test organism. In a nutrient medium this *Pseudomonas* species tolerates salt very well until a concentration of 0.5 M is reached. These findings agree generally with those of Hotchkiss *(9)* who studied the effect of certain cations upon bacterial growth. This

worker, employing *E. coli* as the test organism, observed that several inorganic salts, including NaCl, which are of common occurrence in the protoplasmic environment of the bacterial cell are non-toxic. She also observed growth stimulation for *E. coli* at the 0.25 M (NaCl) level. Our results show that upon subculture 2.0 M NaCl in peptone solution supported only slight growth for *Pseudomonas*, indicating that this concentration, representing approximately 11.6% NaCl, is relatively inhibitory. When an aliquot of this suspension was plated for counting, a few typical colonies appeared. In the suspensions containing 4.0 M and 3.0 M NaCl, representing 23.2% and 17.4%, respectively, no growth appeared after 96 hours incubation, indicating that levels somewhere above 12.0% NaCl were distinctly bactericidal in effect. It is interesting to note in Figure 3 that the salt concentration equivalent to physiological saline (0.15 M) supported growth equal to the control 1.0% peptone solution. Therefore, on the basis of this and previously reported evidence, it would appear difficult to embrace the theory that physiological saline is, in the strict sense of the word, toxic to bacteria as a whole. Certainly there is no justification for avoiding its use as a diluent for the *Pseudomonas* species because of possible toxic effects.

The suspension of washed membrane in buffered saline failed to reveal any significant or unusual antibacterial activity. It apparently showed growth promoting activity after 2 hours incubation at 27° to 30° C. (Figure 4). Suspensions of unwashed membrane also showed no inhibitory

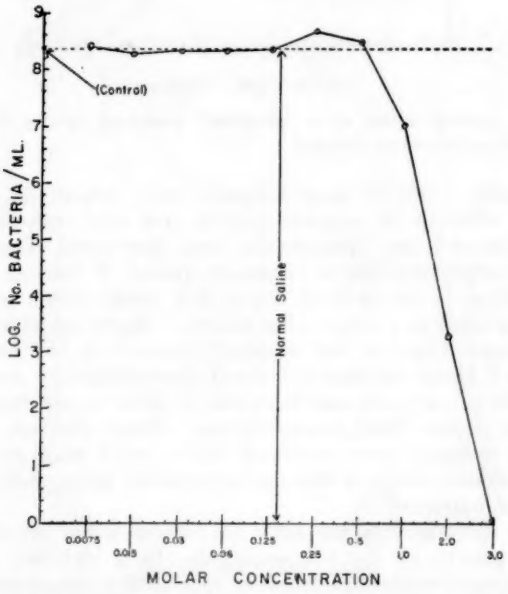

Figure 3. The effect of molar concentration of NaCl in aqueous solution on growth of a "green-rot" producing species of Pseudomonas (PP-H).

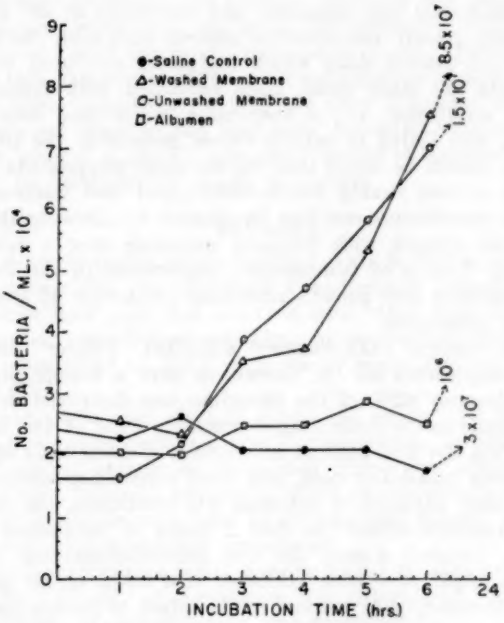

Figure 4. The effect of shell membrane on growth of a "green-rot" producing species of Pseudomonas (PP-H).

action, and after 2 hours incubation growth rates were similar to those in the washed membrane suspension. Albumen in suspension likewise failed to exert any inhibitory activity. The apparent nutritive properties of the shell-membrane are in essential agreement with the findings of Stokes and Osborne *(14)*.

To test the assumption that the membrane might contain antibacterial factors similar to those found in albumen, experiments were performed employing a susceptible organism, *M. lysodeikticus*. The results indicated that lytic effects of unwashed membrane and albumen were immediate and drastic at the time of inoculation and relatively imperceptible thereafter through 6 hours (Figure 5). Washed membrane exerted a killing effect throughout the initial hour of incubation at 37° C., although no drastic reduction in numbers was observed at the time of inoculation. Whether the slope of the washed membrane curve during the first hour was due to the effect of the membrane alone or to any small amount of adhering albumen despite repeated washing was not determined. Variation in the concentration of the inoculum failed to alter significantly the types of curves obtained. An increase in the concentration of washed membrane in the suspension was followed by a corresponding increase in growth. Furthermore, when antibiotic assay discs were impregnated with an uninoculated washed membrane suspension which had been allowed to stand for 3 days, allowed to dry, placed on the surface of an agar plate seeded

with the *Pseudomonas* test organism, and incubated at 30° C. overnight, a zone of intense growth was observed around each disc. No such growth appeared around control discs which had been saturated with buffered saline only. On the other hand, discs saturated with freshly prepared suspension of membrane, i.e., a suspension which had been allowed to stand prior to use, failed to exhibit excess growth at the periphery. In this respect, it should be noted that the chemical components of the shell-membrane are at best weakly water-soluble, and that water-solubilization of the organic constituents can best be effected by allowing the membrane to stand in the diluent with frequent agitation over a relatively long period of time. This is of fundamental importance in the demonstration of growth-promoting and growth-inhibiting properties of a substance by agar-diffusion techniques.

Stokes and Osborne *(14)* observed a limited "killing" effect of membrane-saline suspensions on *Ps. fluorescens* over a 5-hour period, during which approximately 82% of the inoculum was destroyed or inactivated. However, in their saline control approximately 98% of the inoculum was destroyed within the first hour of incubation. On the basis of our results, there is a strong possibility that, had their suspending medium and control been initially adjusted to optimum pH conditions, the drastic reduction in cell numbers within the first 2 hours of incubation might have been avoided. In such a case, the true growth-supporting properties of the membrane might have been exhibited at a much earlier period during incubation. However, with respect to the effect of membrane on the test organisms employed, no basic disagreement is apparent between our results and those of the above workers. Strain differences in test organisms in susceptibility to NaCl may have accounted for dissimilarities. Our

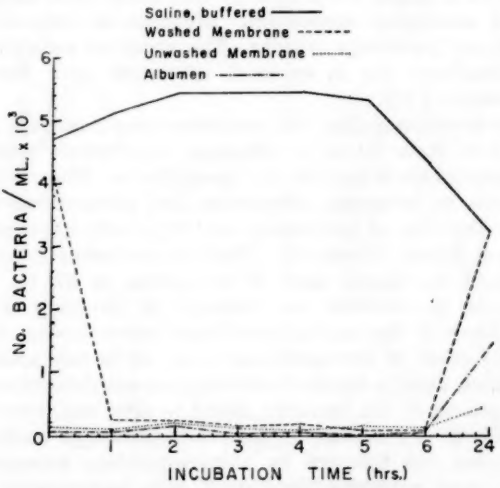

Figure 5. The effect of shell membrane suspension on growth of a lysozyme-sensitive organism (M. lysodeikticus).

observations tend to confirm the findings of Stokes and Osborne *(14)* that albumen-free shell-membrane unquestionably possesses nutritive properties for the organism most commonly implicated in the "green-rot" spoilage of eggs. In fact, the results of the membrane studies of both investigations might have been difficult to explain had they indicated otherwise, since an abundance of organic and inorganic material conducive to, if not essential for, growth of the *Pseudomonas* species of egg-spoilage organisms is known to be incorporated in the membrane.

SUMMARY

Of nine diluents tested, saline buffered with M/15 Na_2HPO_4 to pH 7.3 maintained a bacterial population of *Pseudomonas* organisms at a more nearly constant level than was observed with other suspending media.

Growth of a green-fluorescent pigment producing strain of *Pseudomonas* was maintained in NaCl solutions of concentrations ranging from 0.0075 M to 0.5 M.

The growth promoting effect of a buffered saline (pH 7.3) suspension of albumen-free shell membrane was demonstrated. Growth rate in suspensions of washed membrane, unwashed membrane, albumen, and buffered saline were followed by hourly plate counts over a 6-hour period. Agar-diffusion methods were used to demonstrate intense growth around discs saturated with membrane suspension.

The influence of antibacterial factors found in albumen on any possible inhibitory activity of membrane was determined. A lysozyme-susceptible organism, *M. lysodeikticus*, was employed.

Varying cell concentration of the inoculum failed to affect the results.

The true ability of the membrane to promote growth of spoilage organisms can be masked by the use of inadequate suspending media.

LITERATURE CITED

1. Brooks, M. Comparative studies on respiration. VIII. The respiration of *Bacillus subtilis* in relation to antagonism. *J. Gen. Physiol.*, **2**, 5 (1919).
2. Butterfield, C. T. The selection of a dilution water for bacteriological examinations. *J. Bact.*, **23**, 355 (1932).
3. Eisenberg, P. Untersuchen über spezifische Disinfektionsvorgange. II. Mitteilung: über die Wirkung von Salzen und Lonen auf Bakterien. *Centralblatt Bakteriologie*, **82**, 69 (1918).
4. Elliott, R. P. Spoilage of shell eggs by *Pseudomonads*. *Appl. Microbiol.*, **2**, 158 (1954).
5. Gillespie, J. M., and Scott, W. J. Studies in preservation of shell eggs. IV. Experiments in the mode of infection by bacteria. *Aust. J. Appl. Sci.*, **1**, 514 (1950).
6. Greaves, J. E. The influence of salts on the bacterial activities of the soil. *Soil Science*, **2**, 443 (1916).
7. Gustafson, F. G. Comparative studies on respiration. IX. The effects of antagonistic salts on the respiration of *Aspergillus niger*. *J. Gen. Physiol.*, **2**, 17 (1919).
8. Holm, G. E., and Sherman, J. M. The salt effects in bacterial growth. I. Preliminary paper. *J. Bact.*, **6**, 511 (1921).
9. Hotchkiss, M. Study on salt action. IV. The stimulating and inhibitive effect of certain cations upon bacterial growth. *J. Bact.*, **8**, 141 (1923).
10. Lorenz, F. W., Starr, P. B., Starr, M. P., and Ogasawara, F. X. The development of *Pseudomonas* spoilage in shell eggs. I. Penetration through the shell. *Food Research*, **17**, 351 (1952).

11. MALLMAN, W. S., AND DAVIDSON, J. A. Oil protected shell eggs. U. S. Egg and Poultry Mag., **50**, 113 (1944).
12. MILLER, W. A., AND CRAWFORD, L. B. (Unpublished data, Dept. Bacteriology, Kansas State College, 1948).
13. ROMANOFF, A. L., AND ROMANOFF, A. J. *The Avian Egg.* pp. 144, 322. 1949. John Wiley and Sons, Inc., New York.
14. STOKES, J. L., AND OSBORNE, W. W. Effect of the egg shell membrane on bacteria. *Food Research*, **21**, 264 (1956).
15. STOKES, J. L., OSBORNE, W. W., AND BAYNE, H. G. Penetration and growth of *Salmonella* in shell eggs. *Food Research* (in press).
16. STUART, L. S., AND MCNALLY, E. H. Bacteriological studies on the egg shell. U. S. Egg and Poultry Mag., **49**, 28 (1943).
17. WILSON, G. S. The proportion of viable bacteria in young cultures with an especial reference to the technique employed in counting. *J. Bact.*, **7**, 405 (1922).
18. WINSLOW, C. E. A., AND FALK, I. S. The effect of mineral salts upon the viability of bacteria in water. *Abst. Bact.*, **3**, 5 (1919).
19. ZAGAEVSKY, J. S., AND LUTIKOVA, P. O. Bacterial contamination in eggs. U. S. Egg and Poultry Mag., **50**, 75 & 88 (1944).

A MODIFIED LATIN SQUARE DESIGN FOR TASTE-TESTING

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The problem of intercomparing five or six different varieties of a product as regards some organoleptic characteristic often arises in research laboratories. The products may differ slightly in composition, in source of ingredients, in method of processing; and the quality to be compared may be impossible to evaluate by objective means, such as acceptability of flavor. At such times the research officers resort to the laboratory analytic panel for detection of *differences*. Such panels function as a piece of equipment for measuring the quality concerned as accurately as possible and have nothing to do with *preferences* which are the job of consumer-panels (*6*). Furthermore, it must be understood that their findings must not be unduly generalized.

Once this is clear, the fundamental statistical procedures are applicable. These consist of selecting an expert panel, a suitable scoring scale, a design which controls all foreseeable sources of error, and the appropriate method of analysis. The statistical requirements for independent judgments, tasting the products in a given order, and other requirements of the design must be met during the operating routine of the panel, as well as the mechanical details for optimum operation (*2*, *8*).

The selection of an expert panel is usually made by use of multiple choice tests with the product involved [see p. 12, Dawson and Harris (*2*)]. A panel of three to six is usually sufficient for the purpose described. The scoring scale is also developed by preliminary work with the product. The experimental design has to take into account variation from sitting to sitting (non-reproducibility), variation for the panel during sittings (fatigue), and as many other controllable sources of variation as possible. Variation among judges need *not* be controlled, as the quantity analyzed in such cases is the total score, summed over the judges in the panel; the panel as a whole is the measuring instrument, there is no particular interest in the scores of individual judges.

When the results are obtained they are analyzed according to the appropriate method and the underlying hypothesis that "there are no significant differences amongst the products" is either accepted or rejected.

THE EXPERIMENTAL DESIGN

Judges are restricted to tasting no more than 7 samples at a sitting [this figure varies, see p. 33, Dawson and Harris (*2*)]. When the number of varieties to be compared is greater than seven, incomplete block designs become necessary. One is very frequently interested in the intercomparison of 5 or 6 products. In such cases the conventional latin square design is particularly useful. The columns of the latin square may be the sittings (thus controlling non-reproducibility), the rows the positions in

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order of tasting (thus controlling fatigue), and the "treatments" are the varieties of the product to be intercompared. Many previous experiments (*2*) report on similar designs. What is a suitable design in other fields of application can, however, be improved on in the field of taste-testing. One such attempt to improve is by Calvin (*1*). Others depart from conventional methods and try non-parametric methods [see e.g., Durbin (*3*)]. However, when samples are judged for flavor serially, it is the author's belief that the conventional latin square design can be slightly modified to control the errors introduced by the after-taste of (or involuntary comparison with) the sample tasted immediately prior to the one being judged. This modification, or additional requirement, merely means that in selecting a design for a particular experiment not all latin squares will be eligible, but only those latin squares which are "balanced for first-order residual effects"; e.g., whereas the latin square

A B C D
B C D A
C D A B
D A B C

is such that the treatment B always follows A, C follows B, D follows C, and A follows D — i.e., it is *not* balanced for first-order residual effects; the latin square

A B C D
B C D A
D A B C
C D A B

is such that treatment A follows B, C, D, once each, B follows C, D, A, once each, C follows D, A, B, once each, and D follows A, B, C, once each. Hence, in the former design the after-taste of each variety is confounded with the taste of the subsequent one, but not so in the second. When the number of varieties to be compared is odd, two latin squares are required for balance [see Williams (*13*) for discussion of conditions of balance, construction, and list of available designs]. Whether the introduction of this additional feature is worth the extra arithmetical and mechanical labor in the analysis and operation of the panel respectively (as against the conventional latin square) depends on the amount of reduction in the error mean square by use of this design. A study of the analysis of variance tables in the numerical illustrations below will make this clear.

METHOD OF COLLECTION OF DATA

Given the number of varieties, n, to be compared, select an initial column (if n is even) or a pair of initial columns (if n is odd) at random from the set of columns available and listed in Williams (*13*). Assign treatment-symbols to the treatments at random. Assign columns to the dates on which sittings are to be held at random, after developing the full design from the initial column or columns. Obtain panel-totals at each sitting for each variety, using the scoring-scale decided on, and all necessary precaution in panel-operation during judging. It may, despite all precautions, be necessary to transform scores and work with these transformed data from there on. [See e.g., Federer (*4*) for a summary on the subject of transformations.] The entries are thus score-totals for a given variety at a given sitting (summed over judges). The tables and analyses in the examples show what follows next.

UNDERLYING STATISTICAL MODEL

In the relation

$$\eta_{ijkh} = \mu + \rho_i + \gamma_j + \theta_k + \lambda_h + \epsilon_{ijkh}$$

All symbols have the same meaning as in the classical latin square, with λ_h the added feature;

i.e. η_{ijkh} = total score for the panel (summed over the judges) for the ith position in order of presentation at sitting j, when a sample of variety k is in that position at that sitting and a sample of variety h was in the $(i-1)$th

position, with i, j, k, h = 1, 2, . . . , n, except that a given variety occurs once in each row, once in each column and following each other variety once only;

μ = general mean effect; average over all samples, positions in order of tasting, and sittings;

ρ_i = effect due to the ith position in order of tasting;

γ_j = effect due to the jth sitting;

θ_k = effect characteristic of the kth variety;

λ_h = after-effect characteristic of the hth variety;

ϵ_{ijkh} = error in the observation of y_{ijkh}.

For the purposes discussed ρ_i, γ_j, θ_k, λ_h, can all be regarded as fixed effects [this strictly limits conclusions to material included in the experiment; no generalization is possible to other panels, other varieties, etc., — but as long as this is remembered, the analysis below is strictly applicable; to see some of the pitfalls otherwise, consult Kempthorne (*9*), Ch. 29 and earlier]. Further comments on the applicability of this model are given in the Summary (VII) below.

THE METHOD OF ANALYSIS

1. Notation. [as in Williams (*13*)]

n = number of varieties; number of rows [positions in order of tasting]; number of columns [sittings]

S_{ik} = *total* of entries for kth column of those in which variety i occurs last

F_i = *total* of entries for which variety i is last

T_i = *total* of entries for variety i

R_i = *total* of entries for samples immediately preceded by samples of variety i

P_j = *total* of entries for position j in order of tasting

G = grand total of all entries

f_i = *average* effect for all columns with variety i last

t_i = *average* direct effect of variety i (estimate of θ_k in model)

r_i = *average* residual effect of variety i (estimate of λ_h in model)

r_i' = *average* residual effect of variety i with direct effects ignored

t_i' = *average* direct effect of variety i with residual effects ignored

p_j = *average* effect of position j in order of tasting i, j, k, h as in IV above

$A_i = n^2 t_i' = nT_i - G$ [associated divisor for sum of squares: n^2]

$B_i = n(n^2 - n - 1)r_i' + nF_i + nP_i - (n+1)G$
[associated divisor for sum of squares: $n^2(n^2 - n - 1)$]

$C_i = n(n^2 - n - 2)r_i = n^2R_i + nF_i + nT_i + nP_i - (n+2)G = A_i + B_i$ [associated divisor for sum of squares: $n^2(n^2 - n - 2)$]

$D_i = n(n^2-n-2)t_i = (n^2-n-1)T_i + nR_i + F_i + P_i - nG = \frac{1}{n}[C_i + (n^2-n-2)A_i]$
[associated divisor for sum of squares: $n(n^2-n-1)(n^2-n-2)$]

$\bar{y} = \frac{G}{n^2}$

2. Analysis of Variance.

Interest lies in the two F-tests:

(i) $(n-3)\frac{(SS)_D}{(SS)_E}$ compared with the significant value of F (at the prescribed significance level α with $(n-1)$ and $(n-3)(n-1)$ degrees of freedom. This test indicates whether the varieties are significantly different or not.

(ii) $(n-3)\frac{(SS)_R}{(SS)_E}$ compared with the same F as in (i) indicates whether the varieties have significantly different after-effects. It also shows whether the incorporation of the feature additional to the conventional latin square was worthwhile.

TABLE 1

Source of Variation	*Degrees of Freedom*	*Sum of Squares*
Among sittings	$n - 1$	$\frac{\sum_i F_i^2}{n} - \frac{G^2}{n^2} = (SS)_S$
Among positions in order of tasting	$n - 1$	$\frac{\sum_j P_j^2}{n} - \frac{G^2}{n^2} = (SS)_P$
Direct effects (adjusted for residual effects)	$n - 1$	$\frac{\sum_i [n(n^2 - n - 2)t_i]^2}{n(n^2 - n - 1)(n^2 - n - 2)} = (SS)_D$
Residual effects (unadjusted)	$n - 1$	$\frac{\sum_i [(n^2 - n - 1)r_i']^2}{n(n^2 - n - 1)} = (SS)_{R'}$
Direct effects (unadjusted)	$n - 1$	$\frac{\sum_i T_i^2}{n} - \frac{G^2}{n^2} = (SS)_{D'}$
Residual effects (adjusted for direct effects)	$n - 1$	$\frac{\sum_i [(n^2 - n - 2)r_i]^2}{n(n^2 - n - 2)} = (SS)_R$
Error	$(n - 3)(n - 1)$	$\begin{cases}(SS)_T - (SS)_P - (SS)_{D'} - (SS)_R = (SS)_E \\ (SS)_T - (SS)_P - (SS)_D - (SS)_{R'} = (SS)_E\end{cases}$
Total	$n^2 - 1$	$\sum_i \sum_j \sum_k \sum_h (y_{ijkh} - \bar{y})^2 = (SS)_T$

NUMERICAL ILLUSTRATION

1. The Data.

   A panel of five judges scored for flavor-acceptability the several varieties of canned peas on a 0-10 scale previously tested. The entries for the experiment consisted of total scores over 5 judges. Scores were not transformed. There were 5 varieties. Two latin squares were necessary, the pair being balanced for first-order residual effects. (When the number of varieties to be compared is even, balance over one latin square is possible. For this reason, one often adds an extra variety to the experiment when the original number of varieties is odd to save having to run two latin squares. This extra variety may actually be a "dummy," e.g., a repetition of one of the original ones.) Ten sittings were held, occupying 5 afternoons on each of 2 successive weeks, commencing at 3:00 p.m. One of the degrees of freedom (among sittings) can be regarded as that due to difference between latin squares (or between weeks).
2. The Designs and Analyses.
3. The Conclusions and Discussion.
   (i) The varieties differed significantly.
   (ii) Though the residual effect was not significant, the incorporation of control on residual effects reduced the error mean square appreciably. The modified design can be seen to have justified itself.

(iii) Fatigue effects were significant (within sittings); i.e., the tendency was for the variety tasted first on any afternoon to score higher than the second, it in turn higher than the third, etc. The relation was not far from linear.

(iv) There was evidence of fatigue from sitting to sitting, i.e., the first day's scores were higher than on the second day, etc. Scores were higher in the first week than in the second week.

It has to be noted that sittings are confounded with residual effects, but the statements in (iv) were made after correcting for this.

TABLE 2
Design of the experiment

Sitting No. and Date:	1st week					2nd week				
	6/11	6/12	6/13	6/14	6/15	6/18	6/19	6/20	6/21	6/22
	1	2	3	4	5	1	2	3	4	5
Position in Order of Tasting										
1	3	0	2	4	1	3	0	2	4	1
2	0	2	4	1	3	4	1	3	0	2
3	4	1	3	0	2	1	3	0	2	4
4	2	4	1	3	0	2	4	1	3	0
5	1	3	0	2	4	0	2	4	1	3

TABLE 3
Results of the experiment

(a) First Week

Sitting	1	2	3	4	5	Total
Position in Order of Tasting						
1	48.0	45.5	41.0	42.5	38.0	215.0
2	44.3	40.2	42.4	30.5	45.8	203.2
3	41.6	36.9	45.1	43.4	37.3	204.3
4	39.2	41.5	35.4	43.1	41.4	200.6
5	36.4	43.5	42.0	35.9	37.8	195.6
Total	209.5	207.6	205.9	195.4	200.3	1018.7

(b) Second Week

Sitting	1	2	3	4	5	Total
Position in Order of Tasting						
1	46.4	43.5	39.0	40.5	36.4	205.8
2	40.2	37.5	44.4	41.9	37.8	201.8
3	36.0	44.3	40.6	36.1	38.4	195.4
4	37.8	38.1	33.6	42.3	39.0	190.8
5	40.8	35.5	37.8	38.7	40.0	192.8
Total	201.2	198.9	195.4	199.5	191.6	986.6

TABLE 4
Computations for the experiment

			A_i	B_i	C_i	D_i
In Terms of Estimates			$50t_i'$	$190r_i'$	$180r_i$	$190t_i$
In Terms of Totals	R_i	F_i	$5T_i-G$	$25R_i+5F_i+5P_i-6G$	A_i+B_i	$\frac{1}{5}(18A_i+C_i)$
i = 0	309.9	407.1	106.7	−144.8	− 38.1	376.5
1	330.4	409.0	−208.3	377.2	168.9	−716.1
2	323.0	394.3	−106.3	118.7	12.4	−380.2
3	310.5	399.2	209.2	−169.3	39.9	761.1
4	310.7	395.7	− 1.3	−181.8	−183.1	− 41.3
Total	1584.5					
Add P_i	420.8					
Total	2005.3	2005.3	0.0	0.0	0.0	0.0
Divisor for Sum of Squares			250	4750	4500	3420
Sum of Squares			439.36	50.33	14.50	403.53

TABLE 5
Analysis of variance for the experiment

Source of Variation	Degrees of Freedom	Sum of Squares: Direct Effects Adjusted*	Sum of Squares: Residual Effects Adjusted*	Mean Squares (Adjusted)
Sittings	9	59.18		6.58
Between groups	4	17.74		4.44
Within groups	5	41.44		8.29
Between weeks	1	20.61		20.61
Within weeks	4	20.83		5.21
Positions in Order of Tasting	4	66.07		
Direct Effects	4	403.53	439.36	100.88
Residual Effects	4	50.33	14.50	3.63
Error	28	54.78		1.96
Total	49	633.89		

* Only underlined figures were adjusted.

TABLE 6
Table of means for the experiment

Sample No.	Adjusted Mean	Direct Effect (adjusted)	Residual Effect (adjusted)
0	42.09	+1.98	− .21
1	36.34	−3.77	+ .94
2	38.11	−2.00	+ .07
3	44.12	+4.01	+ .22
4	39.89	− .22	−1.02
Standard Error of Difference:		.65	.74

SUMMARY

The author has had experience with the above type of design for $n = 4$, 5, and 6, particularly with $n = 6$. The quality judged was mostly flavor-acceptability and mouthfeel, the material canned peas, peaches, apricots, apple-sauce, and corn. In a large proportion of some 84 analyses, the mean square for residual effects has been barely or nearly significant at the $\alpha = .05$ level, and in only two instances was the mean square for residual effects less than the error mean square.

In judging color, uniformity, and other characteristics, which can be done by simultaneous inspection of a number of samples it has been found that the above model cannot compete with that proposed by Calvin (*1*), but does better on the flavor and mouthfeel scores than the latter. The suggested explanation for this is as follows: when a characteristic is being judged by simultaneous inspection, all the samples set out at that time influence the score given to any one, but when samples are savored serially, the operating mechanism is one of subconscious comparison of successive samples. As Calvin's (*1*) model incorporates a mechanism geared to the former and the author's (*5*) and Williams' (*13*) design is adapted to the latter, it is not surprising that this should be the case. It also explains why samples tasted earlier than immediately before a given one do not influence its score appreciably. This was verified by using designs on occasion which were balanced for second-order residual effects [see Williams (*14*)]: residual effects of order higher than the first were in no case significant. There is also no evidence that the direct effect of one variety interacts with the residual effect of another *in such experiments as described,* but one suspects that if two or more of the primary taste concentrations were to vary violently from variety to variety that the model would need to incorporate an interaction term.

This discussion has been an attempt to justify the use of the model. The applicability of such models can sometimes be tested by internal checks, sometimes by external checks, and most often not at all. Features new to this model have been singled out in the discussion; other features (e.g., additivity) have not been specifically mentioned. Whereas the results and analysis can never prove a model to be applicable, in the case of this model there is no evidence to the contrary.

Patterson (*10, 11, 12*), has used similar models in incomplete blocks, of which the latin square is a special case, useful because of its balance and facility in computation; there is, of course, no reason why incomplete blocks should not be used when the number of varieties is too great for a latin square. For other historical information on this model see Ferris (*7*).

It is pointed out by Kempthorne (*9*) that there is a tendency to incorrectly analyze such data as the above as a split plot — i.e., one regards the judges as random representatives of the population of consumers (which they of course are not, as they are experts in the laboratory) and breaks the total panel-score which is the whole-plot entry above into the individual judges' scores; one then follows the formal split-plot analysis (see his Ch. 29). This can be (and was) done as a matter of interest, but one *cannot generalize* conclusions from such an analytic panel to the consumers at large.

The design serves a specific purpose and serves it well. The underlying model is a slight variation on classical statistical models used in other fields. The author feels that similar modifications of existing models will enable more efficient statistical analyses to be carried out for other problems arising in taste-testing.

LITERATURE CITED

1. Calvin, L. D. Doubly balanced incomplete block designs for experiments in which treatment effects are correlated. *Biometrics*, **10**, 61, (1954).
2. Dawson, Elsie H. and Harris, Betsy L. Sensory methods for measuring differences in food quality. Bureau of Human Nutrition and Home Economics, U.S.D.A., *Ag. Inf. Bul.*, **34**, (1951).
3. Durbin, J. Incomplete blocks in ranking experiments. *Brit. J. Psych. (Stat. Sect.)*, **4**, 85, (1943).
4. Federer, W. T. *Experimental Design: Theory and Application.* 1956. Macmillan Company, New York, N.Y.
5. Ferris, G. E. Tasting tests and the statistician. (Paper read at Melbourne meeting of Australasian region of the Biometric Society, January, 1948.)
6. Ferris, G. E. Taste panels. Publication BU-61-M, Biometrics Unit, Cornell University, (1956).
7. Ferris, G. E. Statistical designs in taste-testing. (Unpublished Ph.D. Thesis, Cornell University, 1957.)
8. Hopkins, J. W. Instructions as to procedure in laboratory tasting. *Can. J. Research*, **XXIV** and **XXV**, No. 1, (January, 1946).
9. Kempthorne, O. *The Design and Analysis of Experiments.* 1952. Wiley, New York, N.Y.
10. Patterson, H. D. The analysis of change-over trials. *J. Agric. Sci.*, **40**, Part 4, 375, (1950).
11. Patterson, H. D. Change-over trials. *J. Royal Stat. Soc.*, **13**, No. 3, 256, (1951).
12. Patterson, H. D. The construction of balanced designs for experiments involving sequences of treatments. *Biometrika*, **39**, 32, (1952).
13. Williams, E. J. Experimental designs balanced for the estimation of residual effects of treatments. *Australian J. Sci. Res.*, **2**, No. 2, Series A, 149, (1949).
14. Williams, E. J. Experimental designs balanced for pairs of residual effects. *Australian J. Sci. Res.*, **3**, No. 3, Series A, 351, (1950).

SOME STATISTICAL CONSIDERATIONS IN ORGANOLEPTIC RESEARCH: TRIANGLE, PAIRED, DUO-TRIO TESTS

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The purpose of this paper is to explain and demonstrate an improved approach for determining sample sizes and critical regions for triangle, paired, and duo-trio tests. The calculations are made prior to conducting these tests. The new calculating procedure will be presented and discussed including a discussion of its advantage over current procedures. The paper will not deal with any experimental design considerations other than the statistical considerations of necessary sample size and critical region boundaries.

It is presupposed the reader has an acquaintance with the statistical concepts involved in hypothesis testing. Although reviewed to some extent in this paper, the concepts of type I and type II errors are assumed to be understood *(1, 4)*. Also the basis for choosing between one- and two-tailed hypothesis tests should be understood *(7)*. The references indicated above cite recent articles covering the topics of type I and type II errors and one and two-tailed tests respectively. Statistical inference texts also treat these concepts *(3, 6)*. Finally, background information specifically pertinent to this paper can be found in Chapter 2 of Kempthorne's *Design and Analysis of Experiments (5)*.

Previous consideration has been given to sample size determination in triangle ranking tests *(7)*, paired and duo-trio tests *(7)*, and triangle tests *(8)*. Viewed from a statistical hypothesis testing viewpoint, the sample size determination procedures cited above are incomplete, for the type II error or error of the second kind is ignored. They consider only the type I error. The procedure presented in the remainder of this paper considers both type I and type II errors.

SAMPLE SIZE AND CRITICAL REGION DETERMINATIONS

An example will be used to demonstrate the calculations involved in determining the necessary sample size and the critical region to insure certain α and β sizes. The various symbols used in the calculations are listed and defined in the glossary section. The term α is the probability of committing a type I error; that is, α is the probability of rejecting the hypothesis when in fact it is true. The term β is the probability of committing a type II error; that is, β is the probability of accepting the hypothesis when in fact it is false. Suppose a paired test is used to determine the taste preference between two similar products called A and B. The null hypothesis stating there is no taste preference between the two products, A and B, is to be tested. This hypothesis can be stated statistically as

$$p_1' = 0.50.$$

Here p_1' is defined as the expected proportion of A sample preferences to total pairs presented; q_1' is the expected proportion of B sample preferences to total pairs presented. The alternate hypothesis states there is a taste preference between product A and product B. Statistically the alternate hypothesis can be stated as

$$p_2' \neq 0.50.$$

Here p_2' is the expected proportion of A sample preferences to total pairs presented. This is the same definition used for p_1'. The subscripts 1 and 2 on p' differentiate between expected proportions from the hypothesis and the alternate hypothesis respectively.

In order to proceed with the statistical design of this experiment, several choices must be made. These choices are made taking into account the purpose of the experiment and the time, personnel, and financial resources available for the research. First a choice must be made between a one- and two-tailed hypothesis test *(7)*; in this example a two-tailed hypothesis test will be used since there is interest in the preference of A over B and also interest in the preference of B over A. If there is a preference for A over B, the observed proportion of A preference to total pairs, called p, will be larger than 0.50; if, however, there is a preference for B over A, p, the observed proportion of A preferences to total pairs will be less than 0.50.

The second and third choices to be made concern the magnitude of the probabilities of committing errors of type I and type II, α and β respectively. Here an $\alpha = 0.05$ and a $\beta = 0.10$ will be used.

The fourth and last choice to be made is to decide on the difference in proportions that would be considered large enough to be of practical interest or value. As stated previously, it has been hypothesized that there is no taste preference between products A and B, which was stated statistically as

$$p_1' = 0.50.$$

A question arises. If the alternate hypothesis, $p_2' = 0.51$, were true, that is, if the true preference proportion of A over B were 0.51; would this difference between 0.50 and 0.51 be large enough to be of practical importance? Given a large enough sample size such a small difference could be detected as a statistically significant difference. Thus the experimenter must decide on the size of the difference between the proportions from the hypothesis and alternate hypothesis to be considered a practical or an important difference. Such a difference has been labelled δ *(2)*. Here a δ of 0.15 will be used.

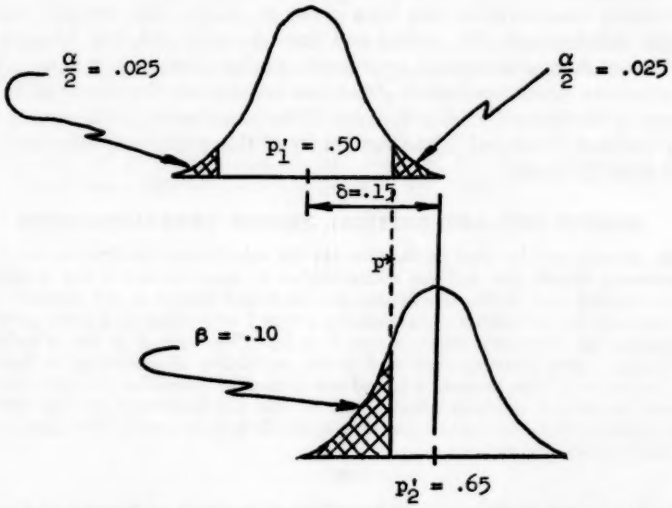

Figure 1. Distribution of sample proportions for the case when the hypothesis ($p_1' = 0.50$) holds and for the case where the alternate hypothesis ($p_2' \neq 0.50$) specifically ($p_2' = 0.65$) is true. The value of $\alpha = 0.05$ has been split into the two tails since a two-tailed hypothesis test is desired. The proportion p* is the upper boundary of the critical region.

Figure 1 graphically portrays the procedure up to this point. The probability distribution centered around $p_1' = 0.50$ represents the distribution of sample proportions that would be obtained if the hypothesis of $p_1' = 0.50$ were true. The probability distribution centered around $p_2' = 0.65$ represents the distribution of sample proportions that would be obtained if the alternate hypothesis $p_2' \neq 0.50$ were true. Note the alternate hypothesis, $p_2' \neq 0.50$ has an infinite number of values; the choice of a specific value for δ pin points the alternate hypothesis at a practical value, 0.65 in this part of the example.

Assuming the sample proportions are binomially distributed and allowing a normal approximation to the binomial, Figure 1 can be translated into two equations,

$$\frac{p^* - 0.50}{\sqrt{\frac{(0.50)(0.50)}{n}}} = +1.960 \tag{1}$$

and

$$\frac{p^* - 0.65}{\sqrt{\frac{(0.65)(0.35)}{n}}} = -1.282 \tag{2}$$

Solving these two equations simultaneously for n and p*, the results $n = 115$ and $p^* = 0.59$ are obtained.

Since the experimental interest is such that a two-tailed hypothesis test was chosen, the above calculations must also be considered for sample proportion distributions centered around $p_1' = 0.50$ and $p_2' = 0.35$. Essentially this means that protection is desired against erroneously accepting the hypothesis, $p_1' = 0.50$, if in fact the alternate hypothesis, $p_2' = 0.35$, is true. The specific value of 0.35 for the alternate hypothesis stems from $\delta = 0.15$ since the difference between 0.50 and 0.15 is 0.35. The equations so formed are

$$\frac{{}^*p - 0.50}{\sqrt{\frac{(0.50)(0.50)}{n}}} = -1.960 \tag{3}$$

and

$$\frac{{}^*p - 0.35}{\sqrt{\frac{(0.65)(0.35)}{n}}} = +1.282 \tag{4}$$

Solving these two equations simultaneously for n and *p, the results $n = 115$ and ${}^*p = 0.41$ are obtained.

Note the sample size is identical to the sample size calculated using equations (1) and (2). Note also the critical region boundaries, *p and p*, are equidistant from 0.50, the hypothesized value of the preference proportion. Thus the formulation and solution of equations (3) and (4) was unnecessary. This is true in the cases where p_1' is hypothesized equal to 0.50, specifically the paired and duo-trio tests. In these cases there is symmetry and the solution of the second set of equations is unnecessary. However, in a two-tailed triangle test both sets of equations must be solved.

Calculations have been made indicating $n = 115$, $p^* = 0.59$, and ${}^*p = 0.41$. The asterisk to the right of p, p*, denotes the boundary of the upper portion of the critical region, and *p denotes the boundary of the lower portion of the critical region. What do these results mean? First of all, 115 preference trials are to be conducted. The actual preference proportion for product A is then calculated. If the preference proportion, p, is less than 0.59 and greater than 0.41, the hypothesis of no taste difference is accepted. If p is equal to or greater than 0.59, or if p is equal to or less than 0.41, the hypothesis is rejected. Using the above accept-reject rule with the stated sample size, the probability of rejecting the hypothesis, $p_1' = 0.50$, when in fact this hypothesis is true will be 0.05 or less (type I error). If the hypothesis is accepted, the probability of erroneously accepting it will be 0.10 or less if in fact p_2' is as large or larger than 0.65 or as small or smaller than 0.35 (type II error).

General equations can be formed corresponding to the ones used in the previous example.

$$\frac{p^* - p_1'}{\sqrt{\frac{p_1' q_1'}{n}}} = Z_\alpha \text{ or } Z_{\alpha/2} \tag{5}$$

and

$$\frac{p^* - p_2'}{\sqrt{\frac{p_2' q_2'}{n}}} = Z_\beta \tag{6}$$

The Z values or right hand sides of equations (5) and (6) are obtained from normal curve tables and are dependent on the choices made for α and β. In equation (5) Z_α is appropriate for a one-tailed test and $Z_{\alpha/2}$ is appropriate for a two-tailed test. Appropriate substitutions into the left hand side of equation (5) are determined from the distribution of sample proportions expected if the hypothesis were true; thus p_1' is the hypothesized proportion. In the paired and duo-trio tests p_1' will be equal to 0.50. In the triangle test p_1' will be equal to 0.33 or $\frac{1}{3}$. Equation (6) is formulated from the distribution of sample proportions expected if the alternate hypothesis were true; thus p_2' is the proportion given in the alternate hypothesis. But the alternate hypothesis has many values; a specific value(s) for the proportion(s) contained in the alternate hypothesis is a function of δ. That is $p_2' = p_1' + \delta$ for a one-tailed test and $p_2' = p_1' \pm \delta$ for a two-tailed test. This demonstrates that two sets of two simultaneous equations each must be considered for two-tailed tests; one set from p_1' and $p_2' = p_1' + \delta$ and the second set from p_1' and $p_2' = p_1' - \delta$. In all cases q' can be found using the relationship $q' = 1 - p'$. These equations are solved for p^* or p^* and $^*p$ thus defining the critical region used to test the hypothesis. Also a solution(s) is obtained for n, the number of trials to be conducted. So if an experimenter calculates these values and uses the required sample size and accepts or rejects according to p^* (one-tailed test) or p^* and $^*p$ (two-tailed test), he is assured of an α and a β not exceeding his stated values. The accept-reject rules can be summarized as follows: For a two-tailed test accept the hypothesis if $^*p < p < p^*$, and reject the hypothesis if $p \leq {}^*p$ or if $p \geq p^*$. For a one-tailed test the hypothesis is accepted if $p < p^*$ and the hypothesis is rejected if $p \geq p^*$. When the hypothesis is rejected, the experimenter assumes or operates as if the alternate hypothesis were true.

DISCUSSION

In the following discussion reference will be made to populations and trials; hence it is appropriate to define these terms as used here. A population is the collection of total trials of interest and about which an inference is to be made. In the paired test, a trial consists of two different types of samples; ordinarily the preference between the two types of samples is to be evaluated. In the triangle test, a trial consists of three samples, two of the same type and one of a different type; the difference between the two types of samples is to be evaluated. In the duo-trio test, a trial consists of three samples, first a standard sample followed in random order by another standard sample and a non-standard sample; the difference between the standard type samples and the non-standard type samples is to be evaluated.

The procedure that has been presented here hinges on an assumption that the preference and difference proportions obtained in paired, duo-trio, and triangle testing are binomially distributed. This assumption is widely accepted *(1, 5)*. These proportions will be binomially distributed if the proportions were obtained from a population such that the population preference probability or difference probability is constant. It follows from the type of null hypothesis stated in this paper that the population preference probability or difference probability will be constant. Thus the binomial assumption is valid. For example, in using the triangle test to investigate

differences, the null hypothesis states no difference exists between the two types of samples; or stated statistically, the probability of correctly labelling the odd sample from the three samples in a given trial is $\frac{1}{3}$. Under the framework of the null hypothesis, this probability of $\frac{1}{3}$ is known and constant which justifies the use of the binomial distribution in dealing with the obtained proportions. So the procedure reported here is specifically applicable to situations where it is hypothesized that no preference or difference exists in the population, and an inference is to be made from the experimental results concerning population preferences or differences.

A procedural point that also merits further discussion is the use of the normal distribution to approximate the binomial distribution. The following rule (3) can be used to check the appropriateness of the normal approximation to the binomial. If np' is greater than five ($np'>5$), the ratio $p^*-p'/\sqrt{\frac{p'q'}{n}}$ has a reasonably normal distribution. In the context of the present paper, this rule will be met most if not all of the time since p' values will be $\frac{1}{2}$ for duo-trio and paired tests and $\frac{1}{3}$ for triangle tests. Applying this rule to the previous example $np_1' = (115)(0.50) = 57.5$ for the proportion derived from the hypothesis, and $np_2' = (115)(0.35) = 40.25$ and $np_2' = (115)(0.65) = 74.75$ for the specific proportions derived from the alternate hypothesis. The use of the normal approximation was quite appropriate in this example. It is suggested that this calculation be performed as a check especially for n's less than 50 or 60.

It was stated originally that hypothesis tests ignoring the type II error are incomplete, and that the procedure presented here represented an improvement by considering both type I and type II errors.

An example of the importance of the type II error is found in duo-trio tests conducted to determine if the current batch of product tastes the same as the standard product. Hypothesizing there is no taste difference between current production and the standard product, committing the type I error involves labelling current production as differing from the standard when in fact it is the same. Unnecessary corrective action might be taken by further blending, processing, or even discarding the current batch. On the other hand, committing the type II error involves labelling the current production as tasting the same as the standard product when in fact it does not. This current production is then distributed to the consumers, some of whom will detect a shift in taste, and this is precisely what the duo-trio test in this case is designed to prevent. Note committing the type I error results in an internal mistake while committing the type II error affects the consumers' reactions. Both errors are of consequence and should be considered in the experimental design.

Perhaps the best way to demonstrate the incomplete nature of experimental designs ignoring the type II error is to plan an experiment based on a consideration of the type I error only and then investigate the size of β at some specific value of the alternate hypothesis. Suppose a one-tailed triangle test ($p_1' = 0.33$) is to be conducted and the α level is set at 0.01. At this point the experimenter chooses a convenient sample size of say 100 in this example, and calculates the boundary of the critical region. Using notation

and procedure parallel to that used earlier, the following equation is formed by substituting appropriately into equation (5),

$$\frac{p^* - 0.33}{\sqrt{\frac{(0.33)(0.67)}{100}}} = 2.327 \tag{7}$$

Note a set of two equations is not formed since the type II error and the alternate hypothesis have been ignored. Note also that the sample size is arbitrarily chosen and not calculated. Solving the above equation, $p^* = 0.44$. Thus if, p, the observed proportion of odd samples actually labelled odd equals or exceeds 0.44, the hypothesis would be rejected; if p was found to be less than 0.44 the hypothesis would be accepted. The design protects the experimenter against erroneously rejecting the hypothesis, but what are the chances of erroneously accepting the hypothesis when an alternate hypothesis is true? For instance, one would guess that if an alternate hypothesis, $p_2' = 0.46$, were true, the design described above would almost never accept the hypothesis, $p_1' = 0.33$. Substituting into equation (6),

$$\frac{0.44 - 0.46}{\sqrt{\frac{(0.46)(0.54)}{100}}} = Z_\beta \tag{8}$$

and solving equation (8), $Z_\beta = -0.401$. Using a normal curve-table, $\beta = 0.34$. So this design would erroneously accept the hypothesis, $p_1' = 0.33$, about 34 times out of 100 when in fact the alternate hypothesis, $p_2' = 0.46$, was true. This is an obviously weak statistical design.

Had equations (5) and (6) been used with a choice of $\alpha = 0.01$, $\beta = 0.05$, and $\delta = 0.13$, an $n = 217$ and a $p^* = 0.40$ would have been indicated. Although the sample size is a little more than double, the probability of committing a type II error would have been reduced from 0.34 to 0.05.

SUMMARY

A procedure for planning or designing the statistical phases of organoleptic experiments has been presented. The use of the binomial distribution and the normal approximation to the binomial are discussed. Examples demonstrating the advantage of this procedure have been given.

Acknowledgment

The assistance and encouragement given to the author by Dr. C. R. Hicks of the Purdue Statistics Laboratory in writing this paper is greatly appreciated.

Glossary of Symbols

α = probability of committing a type I error
β = probability of committing a type II error
n = the number of trials or the sample size
p = an observed proportion
p^* = the boundary of the upper portion of the critical region. p^* forms the basis for accepting or rejecting the hypothesis.
$^*p$ = the boundary of the lower portion of the critical region. $^*p$ along with p^* form the basis for accepting or rejecting the hypothesis. For a two-tailed test $^*p$ and p^* are both necessary to define the critical region.

p' = the expected or long run average proportion. p' used with a 1 subscript, p_1', refers to the proportion expected under the hypothesis. p' used with a 2 subscript, p_2', refers to the proportion expected under the alternate hypothesis.

δ = the difference between p_1' and p_2' which is large enough to be considered a practical or an important difference.

$q' = 1 - p'$

Z = standard variable; $Z = \dfrac{p^* - p'}{\sigma_p}$ where $\sigma_p = \sqrt{\dfrac{p'q'}{n}}$.

Z values are obtained from normal curve tables.

LITERATURE CITED

1. Baker, G. A., Amerine, M. A., and Roessler, E. B. Errors of the second kind in organoleptic difference testing. *Food Research*, **19**, 206 (1954).
2. Davies, O. L. *Design and Analysis of Industrial Experiments*. 1954, Hafner Publishing Company, New York, N. Y.
3. Dixon, W. J., and Massey, F. J., Jr. *Introduction to Statistical Analysis*. 1951, McGraw-Hill Book Company, Inc., New York, N. Y.
4. Gridgeman, N. T. Taste comparisons — two or three? *Food Technol.*, **9**, 148 (1955).
5. Kempthorne, O. *The Design and Analysis of Experiments*. 1952, John Wiley and Sons, Inc., New York, N. Y.
6. Ostle, B. *Statistics in Research*. 1954, The Iowa State College Press, Ames, Iowa.
7. Roessler, E. B., Baker, G. A., and Amerine, M. A. One-tailed and two-tailed tests in organoleptic comparisons. *Food Research*, **21**, 117 (1956).
8. Roessler, E. B., Warren, J., and Guymon, J. F. Significance in the triangle taste tests. *Food Research*, **13**, 503 (1948).

STUDIES ON THE NON-ENZYMATIC BROWNING OF FOODS—ORGANIC ACID-AMINO MODEL SYSTEMS

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For a long time, work on non-enzymatic browning of food products centered around Maillard type reactions. In recent years, however, the importance of other systems is being increasingly felt. Using ion exchange resins and studying growning in different fractions of apricot concentrate, Haas and Stadtman *(2)* showed that the over-all browning is the result of at least four distinct types of reactions: (a) nitrogenous constituents and sugars, (b) nitrogenous constituents and organic acids, (c) sugars and organic acids, and (d) organic acids alone. Model system studies dealing with (a) have been reviewed by Danehy and Pigman *(1)* and Hodge *(3)*. Work on systems dealing with (c) and (d) has been reported by Lewis *et al. (4, 5)* and more recently by Kapur *et al. (6)*. The present paper deals with investigations on browning in organic acid-amino acid model systems. Though low energy systems *(3)*, they are likely to play an important part in the browning of products prepared from highly acidic fruits.

EXPERIMENTAL

Equal volumes of 0.1 M solutions of 11 organic acids (acetic, propionic, oxalic, malonic, succinic, malic, maleic, citric, tartaric, lactic and pyruvic) and 20 amino acids (DL-glycine, L-cysteine, L-lysine(0.01 M), L-arginine, DL-tryptophan, DL-histidine, L-leucine, DL-α-alanine, DL-phenylalanine, DL-glutamic acid, DL-aspartic acid, DL-valine, DL-serine, DL-methionine, L-tyrosine, L-proline, L-asparagine, L-cystine, DL-isoleucine and DL-threonine) were mixed, taking one organic acid and one amino acid at a time, and stored in stoppered pyrex test tubes in an oven maintained at 55° C. ± 1. Control organic acids and amino acids were likewise stored. Since, in the preparation of solutions of some of the amino acids, addition of HCl was found necessary, the same quantity of HCl was added to all amino acid solutions to obtain uniform pH conditions.

During storage, readings for ultraviolet absorption spectra were taken in the region 220-300 mμ at intervals of 5 mμ with a Beckman DU-spectrophotometer after 1, 5 and 10 weeks. Fluorescence was measured after 1 and 6 weeks with a Klett Summerson Fluorimeter using a riboflavin filter (5200 Å) after adjusting the instrument to a scale reading of 200 with 0.1 mg. per cent aqueous solution of quinine hydrogen sulphate. Intensity of visible colour was measured as optical density with Lumetron colorimeter using a blue filter (420 mμ) after 2, 5 and 10 weeks. For brevity, only first and last readings are recorded in Tables 1 and 3. Values for pH were recorded after 10 weeks' storage using a Phillips electric pH meter.

RESULTS AND DISCUSSION

Ultraviolet absorption spectra. In Table 1 are recorded the positions of the peak in the ultraviolet region of the brown precursors involved in organic acid-amino acid complexes. Freshly prepared organic acid solutions show no peak in the region 270-285 mμ but during storage, the peak started developing except in the case of oxalic and malic acids which had no peak even after 10 weeks of storage. Malonic acid showed a peak at 270, 280 and above 300 mμ after 1, 5 and 10 weeks of storage, respectively.

TABLE 1[1]

Position of the peak (mμ) after 1 and 10 weeks' storage in organic acid-amino acid model systems

Organic acids		Acetic acid	Propionic acid	Oxalic acid	Malonic acid	Succinic acid	Malic acid	Maleic acid	Citric acid	Tartaric acid	Lactic acid	Pyruvic acid
Amino acids		260	Nil	Nil	270	Nil	Nil	Nil	Nil	Nil	260	Nil
		278	278	Nil	300	275	Nil	275	275	275	275	275
Glycine	Nil	275	Nil	Nil	270	Nil	Nil	Nil	280	Nil	270	Nil
	275	275	275	Nil	275	275	275	Nil	280	275	275	Nil
Lysine	Nil	Nil	280	Nil	Nil	280	Nil	Nil	280	Nil	Nil	Nil
	Nil	278	275	275	275	275	Nil	Nil	278	278	275	Nil
Arginine	270	260	260	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
	275	273	275	Nil	275	278	275	Nil	275	275	275	Nil
Tryptophan	280	280	280	278	280	280	280	280	280	280	280	275
	280	275	275	278	278	280	278	275	280	278	280	278
Histidine	275	270	270	Nil	270	270	Nil	Nil	260	260	260	Nil
	275	278	275	Nil	275	275	275	Nil	275	273	275	275
Leucine	260	260	Nil	Nil	Nil	260	Nil	Nil	260	Nil	260	Nil
	275	275	280	275	275	275	275	Nil	275	278	275	Nil
Alanine	260	Nil	260	Nil	260	Nil	Nil	Nil	260	Nil	260	Nil
	275	280	270	Nil	275	270	275	Nil	275	275	275	Nil
Phenylalanine	260	260	260	260	260	260	260	Nil	260	260	260	Nil
	260	Nil	260	260	260	260	260	Nil	260	260	260	260
Glutamic acid	270	270	270	Nil	Nil	260	Nil	Nil	275	260	280	Nil
	275	280	275	Nil	275	275	275	Nil	275	275	275	Nil
Aspartic acid	270	275	Nil	275	275	270	275	275	Nil	270	273	Nil
	275	278	278	275	270	275	275	Nil	278	278	270	Nil
Valine	270	260	270	Nil	270	260	260	Nil	Nil	Nil	Nil	Nil
	275	270	275	Nil	280	275	275	Nil	275	275	275	Nil
Serine	Nil	280	280	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
	275	275	275	Nil	278	275	275	Nil	278	275	275	Nil
Methionine	Nil	Nil	Nil	Nil	260	Nil	Nil	Nil	Nil	Nil	Nil	Nil
	275	275	275	Nil	280	Nil	280	Nil	280	275	280	Nil
Tyrosine	275	275	275	275	275	275	275	275	275	275	275	275
	275	275	275	280	275	275	275	275	275	275	275	275
Proline	Nil	275	275	Nil	270	Nil	Nil	Nil	Nil	270	Nil	Nil
	275	275	275	275	275	275	275	Nil	275	275	Nil	275
Asparagine	Nil	270	260	Nil	270	Nil	Nil	Nil	Nil	Nil	Nil	Nil
	273	278	280	Nil	275	275	275	275	275	275	275	Nil
Isoleucine	260	Nil	260	Nil	Nil	270	Nil	Nil	Nil	270	Nil	Nil
	260	275	275	Nil	280	275	275	Nil	275	275	275	275
Threonine	Nil	270	Nil	Nil	Nil	Nil	Nil	270	260	270	270	Nil
	275	275	273	Nil	278	Nil	275	275	275	270	275	Nil

[1] First vertical column gives the peak for pure amino acids. First horizontal column indicates the peak for pure organic acids. The values in other columns relate to the systems.

After 17 weeks, the peak was at 300 mμ in freshly prepared amino acid solutions; however, a peak in the region of 270-285 mμ was shown by arginine, tryptophan, histidine, leucine, alanine, phenylalanine, glutamic acid, aspartic acid, valine, tyrosine and isoleucine, while glycine, cysteine, lysine, serine, methionine, proline, asparagine, cystine, and threonine developed a peak at 275 mμ during storage. No peak was visible in cysteine, lysine and cystine even after 10 weeks of storage.

In organic acid-amino acid systems, the complexes involving cystine-organic acids and cysteine-organic acids showed no peak at any stage during storage, indicating thereby the inhibitory effect of S—S. and —SH— linkage present in cystine and cysteine, respectively. The reducing action of these groups probably prevents the conversion of reductones into more reactive dehydro forms *(3)*. Methionine which contains sulphur in the bound form ($-S-CH_3$) showed no such effect.

The inhibition of development of peak in organic acid-amino acid systems was noted in pyruvic and maleic acids. In pyruvic acid-amino acid systems there was no peak at any stage during storage in 14 out of 20 amino acids. Of the remaining six, phenylalanine, proline and isoleucine showed peak at 260-275 mμ after 10 weeks, histidine after 5 weeks, and tryptophan and tyrosine at all stages. Presence of an indole ring in tryptophan and a phenolic group in tyrosine seems to contribute towards the development of peak at 275 mμ. In maleic acid-amino acid systems, 16 out of 20 amino acids developed no peak at any stage during storage, while tryptophan, tyrosine and threonine showed it at all stages. The peak at 275 mμ in aspartic acid-maleic acid system shown after one week disappeared during further storage. In malic acid-amino acid systems, there was no peak in 15 out of 20 amino acids after 2 weeks' storage, but afterwards the peak developed in most cases. Oxalic acid which produces very severe browning with fructose and sucrose did not show any such effect with 12 out of 20 amino acids while with others the peak developed gradually.

One noteworthy feature of amino acid-organic acid systems is the occurrence of the peak in the vicinity of 275 mμ except in phenylalanine-organic acid systems where it occurs at 260 mμ. It is, therefore, likely that the nature of brown precursors in these systems may be different from those in sugar-organic acid and sugar-amino acid systems where the peak is at 282 mμ *(3)*. Treatment with a solution of 2:4 dinitrophenyl hydrazine sulphate developed yellow to brown turbidity in all systems showing peak indicating thereby that some carbonyl compounds are involved in these precursors. This is in agreement with Hodge's *(3)* views on the subject.

The values of optical density at peak positions, generally speaking, steadily increase during storage. In some systems, however, where the original peak at 260 mμ disappears slowly, giving place to peak at 275 mμ, the value of the optical density at 260 mμ gradually decreases and that at 275 mμ increases. Another noteworthy point is that only in two systems (acetic acid- amino acids and propionic acid-amino acids), the initial peak occurs at 280 mμ in some cases which subsequently shifts to the lower wavelength region accompanied by a decrease in the optical density.

TABLE 2[1]

Fluorescence values of brown precursors involved in organic acid-amino acid complexes during storage after 1 and 6 weeks

Organic acids		Acetic acid	Propionic acid	Oxalic acid	Malonic acid	Succinic acid	Malic acid	Maleic acid	Citric acid	Tartaric acid	Lactic acid	Pyruvic acid
Amino acids		2	3	2	16	64	45	6	14	5	5	31
		50	18	18	13	88	85	6	30	63(1:1)	48	52
Arginine	162	128	136	110	142	128	135	87	83	125	131	124
	225	158	175	132	168	168	163	110	174	158	153	160
Tryptophan	6	48	26	30	36	32	32	36	32	42	42	12
	2	3	11	17	11	32	26	0	0	12	2	0
Histidine	26	20	14	10	16	14	14	6	18	22	14	122
	55	234	300	110	168	300	300	184	175	300	300	270
Serine	33	8	3	0	6	2	4	0	3	3	1	12
	72	72	40	27	68	53	59	19	52	45	52	52
Proline	43	21	21	21	32	18	21	15	23	23	21	23
	60	36	43	33	150	44	41	38	38	43	70	35

[1] First vertical column gives the peak for pure amino acids. First horizontal column indicates the peak for pure organic acids. The values in other columns relate to the systems.

TABLE 3

Values of optical density at 420 mμ of organic acid-amino acid systems after 2 and 10 weeks of storage

Amino acids	Acetic acid	Propionic acid	Oxalic acid	Malonic acid	Succinic acid	Malic acid	Maleic acid	Citric acid	Tartaric acid	Lactic acid	Pyruvic acid
Tryptophan	0.69	0.42	0.41	0.60	0.32	0.38	0.47	0.44	0.30	0.39	1.12
	1.40	1.00	1.80	1.50	1.60	1.25	1.32	1.80	1.68	1.60	2.00
Histidine	—	—	—	—	—	—	—	—	—	—	—
	0.14	0.22	0.18	0.24	0.34	0.23	0.30	0.21	0.35	0.29	0.42
Cystine	—	—	—	—	—	—	—	—	—	—	—
	0.21	0.21	0.29	0.39	0.30	—	0.36	0.32	—	0.30	0.34

Fluorescence. Data in Table 2 show that, in general, the fluorescence values increased during storage except in organic acid-tryptophan systems where it decreased as the brown colour appeared. (All systems showed fluorescence but data are recorded only for those where the value was more than 30 units.) The rise in fluorescence values indicates the formation of brown precursors.

Visible colour. In Table 3 is recorded the optical density at 420 mμ of the coloured solutions only. In all organic acid-tryptophan systems, the colour after 10 weeks was dark brown which, as already explained, is probably due to the presence of indole ring. A slight yellow colour was also developed in histidine-organic acid systems and a faint yellow colour in a few other systems. It is surprising that except in succinic acid-tyrosine system (O.D.—0.66), but in none of the other systems, tyrosine showed the development of colour even though a strong tendency to do so was indicated by marked increase in the values of optical density at the peak position in the ultra violet region. It is possible that in this case a longer period of storage may be needed to give a definite indication of the polymerization of the brown precursors detected by ultraviolet spectroscopy and fluorimetry. Slight development of pale yellow colour in organic acid cystine systems may be due to the presence of some impurities in the amino acid.

pH value. At the end of 10 weeks' storage period the pH values were 1.0 in cysteine, tryptophan and phenylalanine-organic acid systems; 1.2 in leucine, tyrosine and cystine-organic acid systems; 1.3 in lysine, histidine, glutamic acid and aspartic acid-organic acid systems; 1.4 in arginine, alanine and asparagine-organic acid systems; and 1.75 in glycine, valine, serine, methionine, proline, isoleucine and threonine-organic acid systems. These values show that in the ranges studied, pH alone is not the determining factor in the browning of organic acid-amino acid systems.

SUMMARY

Browning in organic acid-amino acid model systems involving 11 organic acids and 20 amino acids has shown that the ultra violet peak of the brown precursors is generally at 275 mμ.

The amino acids containing —S—S— or —SH— linkage (cystine and cysteine) did not develop brown precursors during a 10 week storage period at 55° C.

The amino acids containing an indole ring or phenolic group (tryptophan or tyrosine) induce browning faster than other amino acids.

Pyruvic and maleic acids seem to retard the browning reaction in organic acid-amino acid systems.

Acknowledgment

The authors are highly grateful to Dr. V. Subrahmanyan, Director of the Institute, for his keen interest in this investigation.

LITERATURE CITED

1. Danehy, J. P. and Pigman, W. W. Reactions between sugars and nitrogenous compounds and their relation to certain food problems. *Advances in Food Research*, **3**, 241 (1951).

2. HAAS, V. A. AND STADTMAN, E. R. Deterioration of dried fruits—use of ion exchange resins to identify types of compounds involved in browning. *Ind. Eng. Chem.*, **41**, 983 (1949).
3. HODGE, J. E. Chemistry of browning reactions in model systems. *J. Agr. Food Chem.*, **1**, 928 (1953).
4. LEWIS, V. M., ESSELEN, W. B., JR., AND FELLERS, C. R. Non-enzymatic browning of foodstuffs—production of carbon dioxide. *Ind. Eng. Chem.*, **41**, 2587 (1949).
5. LEWIS, V. M., ESSELEN, W. B., JR., AND FELLERS, C. R. Non-enzymatic browning of foodstuffs—nitrogen-free carboxylic acids in the browning reaction. *Ind. Eng. Chem.*, **41**, 2591 (1949).
6. KAPUR, N. S., BHATIA, B. S., BHATIA, D. S., AND GIRDHARI LAL. Studies on the non-enzymatic browning of foods: Sugar-organic acid model systems (Communicated for publication).

THE RESPONSE OF HAMSTERS TO A NATURAL-TYPE DIET CONTAINING EMULSIFIERS[a]

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With the wide acceptance by the food industry of polyoxyethylene (8) stearate (Myrj 45) and other surface-active, partial fatty acid esters as emulsifying agents, considerable attention has been directed to the study of the nutritional value and safety of these compounds *(1, 2, 3, 7, 8, 12, 13, 14, 20)*. Although most studies emphasize the innocuous effects of Myrj 45 when ingested in experimental diets at extremely exaggerated dosage levels, Schweigert *et al. (23, 27)*, employing purified diets, reported some deleterious effects in hamsters. The hamster is a relative newcomer among experimental animals for prolonged nutritional studies and much remains to be elucidated of its nutritional requirements and normal physiology *(5)*. In addition, it is known to be less uniform in its responses than the rat and is subject to many transmissible diseases *(18, 22, 28)*. It therefore seemed of interest to reevaluate the hamster response to high levels of Myrj 45 under conditions where the basal diet consisted of natural rather than "purified" ingredients. Although the results of these studies were largely negative insofar as demonstrating toxicity was concerned, it seemed warranted to record our observations because of the recent publication *(4, 19)* of apparently contradictory findings which were originally reported at the Ice Cream Hearings *(25)* before the Administrator of the Federal Security Agency in 1952.

The basic composition of the diet fed in our hamster experiments is the same as that used in long-term studies of the nutritional effects of a large series of emulsifiers in rats *(15)*. It was formulated to resemble the American dietary in respect to the proportions of protein, fat, and carbohydrate present and the proportions of protein and fat derived from animal and vegetable sources, while at the same time satisfying the known nutritional requirements of either rats or hamsters. The test materials were incorporated at the expense of the wheat and corn mixture in the ratio in which the latter appear in the diet (i.e., 2:1).

Two studies were conducted. In the first, diets including equivalent levels of Myrj 45 (polyoxyethylene (8) monostearate) were compared with those containing a hydrogenated vegetable oil (Primex), diglycerides and monoglycerides from meat fats and vegetable fats (sold commercially as "Esterine"), and cottonseed oil monoglyceride ("Myverol 18–85"). The test materials were fed at graded dietary levels (0, 5, 10, and 15%) in successive periods of 2 or 4 weeks' duration, the levels being the same for all materials at any given period.

Because of the observation of increased kidney weights and somewhat lower weight gains in the Myrj 45 hamsters compared with those fed

[a] This investigation was supported by a grant from the Atlas Powder Company, Wilmington, Delaware.

Primex (discussed below), it was of interest subsequently to determine whether the feeding of the neutral fat in combination with Myrj 45 might modify this response. Therefore a second study was undertaken with hamsters in which the effects of Myrj 45 and Primex at 20% levels in the diet were compared with those resulting from the combination of 10% Myrj 45 and 10% Primex.

EXPERIMENT I

Procedure. Forty-eight healthy, weanling hamsters of both sexes, purchased from the Lakeview Hamster Colony, Newfield, New Jersey, served as experimental subjects. They were housed individually in wire-mesh cages in an air-conditioned room at $75 \pm 2°$ F., with food and water available ad libitum. The composition of the rations fed in this experiment is shown in the first portion of Table 1.

TABLE 1

Composition of rations

Ingredient	Experiment I				Experiment II		
	Weeks				Weeks		
	1–2	3–4	5–8	9–12	1–10	1–10	1–10
	per cent				*per cent*		
Myrj 45, Esterine or Myverol)	0	5	10	15	10*	20*	—
Primex)					10	—	20
Whole wheat	42	39	35	32	28	28	28
Yellow corn	21	19	18	16	15	15	15
Non-fat milk solids	20	20	20	20	20	20	20
Meat meal	10	10	10	10	10	10	10
Alfalfa meal	2	2	2	2	2	2	2
Dried yeast	1	1	1	1	1	1	1
Dried pork liver	0.48	0.48	0.48	0.48	0.48	0.48	0.48
Celluflour	1	1	1	1	1	1	1
Sodium chloride	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Vitamin B-complex mixture[1]	1	1	1	1	1	1	1
$MnSO_4H_2O$	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Vitamins in oil[2]	1	1	1	1	1	1	1

[1] Providing per 100 grams of ration: Thiamine, 300 γ; riboflavin, 600 γ; pyridoxine, 200 γ; Ca pantothenate, 2 mg; niacin, 5 mg.; choline chloride, 200 mg.; inositol, 100 mg.; p-aminobenzoic acid, 2.5 mg.; biotin, 1 γ; folic acid, 1 γ; cyanocobalamin, 1 γ; liver concentrate, 25 mg.

[2] Providing per 100 grams of ration: Vitamin A, 200 u.; vitamin D, 20 u.; α-tocopherol, 12 mg.; menadione, 100 γ.

* Myrj 45 only.

The hamsters were distributed into 4 groups equilibrated with respect to litter, weight, and sex distribution. All received the basal ration for a 2-week period to permit them to become acclimatized to laboratory conditions. They were then given the diets containing the experimental samples at the following levels: 5% for 2 weeks, 10% for 4 weeks, 15% for 4 weeks, each group continuing on the same test material. The appearance and behavior of the animals were noted daily and body weights recorded weekly during the entire 12-week period.

At the termination of the experiment, the hamsters were sacrificed, and at necropsy the following organs weighed: liver, kidneys, spleen, heart, adrenals, and thyroid glands. Histopathological examinations were made of the liver, kidney, duodenum, ileum and cecum of two male and two female hamsters on each diet; in addition the kidneys of all of the Myrj 45 group were examined.

Results. No abnormalities of behavior or of physical appearance were observed in the hamsters at any time during the 10 weeks of feeding the experimental diets.

Growth responses are summarized in Table 2 and shown graphically in Figure 1. During the period when the 5% levels were being fel, all 4 test groups grew at essentially the same rate. During the two subsequent periods when the 10 and 15% levels

TABLE 2

Summary of growth responses of hamsters in feeding studies with emulsifiers

Test Material	Number of Hamsters and Sex	Emulsifier Level								
		5 Per Cent			10 Per Cent			15 Per Cent		
		Average weights		Net Gain	Average weights		Net Gain	Average weights		Net Gain
		Initial	2 wk.		4 wk.	6 wk.		8 wk.	10 wk.	
		grams			*grams*			*grams*		
Primex (Control)	7M	54.1	74.6	20.5	85.2	97.0[8]	20.7[6]	106.0[5]	110.2[5]	12.8[5]
	5F	50.6	67.4	16.8	78.8	87.2	19.8	94.4	97.6	10.4
	12MF	52.4	71.0	18.7	82.0	92.1[11]	20.3[11]	100.2[10]	103.9[10]	11.6[10]
Myrj 45	7M	54.1	73.1	19.0	79.1	84.1	11.0	88.7	92.6	8.4
	5F	55.2	75.8	20.6	84.4	91.8	16.0	97.4	102.6	10.8
	12MF	54.7	74.5	19.8	82.0	88.0	13.5	93.1	97.6	9.6
Esterine	5M	51.6	72.4	20.8	83.8	91.4	19.0	96.2	100.0[4]	9.8[4]
	7F	51.9	70.1	18.2	77.9	83.6	13.5	90.5[6]	92.0[5]	9.0[5]
	12MF	51.8	71.3	19.5	80.9	87.5	16.3	93.4[11]	96.0[9]	9.4[9]
Myverol 18-85	6M	55.3	75.8	20.5	86.5	93.0	17.2	98.5	103.7	10.7
	6F	50.7	68.3	17.6	75.0	80.8	12.5	85.3	90.3	9.5
	12MF	53.0	72.1	19.1	80.8	86.9	14.8	91.9	97.0	10.1

Superscripts indicate number of "survivors." One male control met with an accidental death; the remaining decreases in group size resulted from the escape of hamsters from their cages.

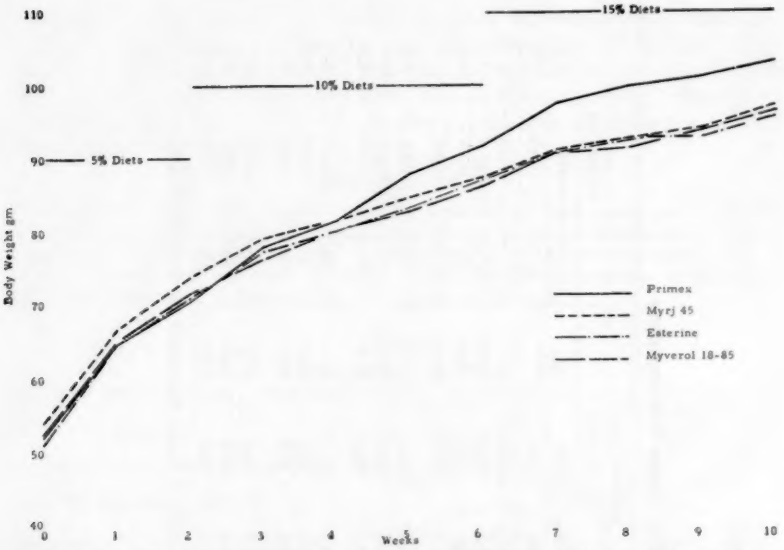

Figure 1. Growth responses of rats fed natural types diets containing Primex, Myrj 45, Esterine, and Myverol 18-85, at progressively increasing levels.

were fed, no significant differences in weight gains were noted among the 3 groups fed Myrj 45, Esterine, or Myverol 18–85, but all grew at a somewhat retarded rate compared with the group fed Primex.

On the basis of the food intake data during the final (15%) period, it was possible to calculate whether this retardation was due to factors other than the limitation imposed by the partial non-calorigenic nature of the test materials. Highest voluntary food intake was in the Myrj 45 group, a manifestation apparently related to the lower energy level of that diet. Caloric values for Primex, Myrj 45, Esterine and Myverol 18–85 were originally assumed to be 9.4, 4.1, 9.0, and 8.7 calories per gram respectively; that of Primex and Myrj 45 were modified by experimentally determined coefficients of digestibility (reported elsewhere from our Laboratories [*16*]) and accordingly the values 8.84 and 3.32 calories per gram were used in computing the caloric densities of these diets.

From the data in Table 3 it may be seen that the efficiency of caloric utilization (ECU) of all the emulsifier diets tended to be lower than that of the Primex diet, the differences being somewhat greater in the males than in the females. In the Myrj 45 group only the male hamsters showed lower ECU values and even in this case the difference was not significant at the $p = 0.05$ level.

No mortality was directly attributable to the experimental diets. One male in the Primex group died as a result of an accidental injury. The Primex and the Esterine groups were further reduced by one and three hamsters respectively who escaped from their cages and were at large several days before recapture.

Diarrhea was not observed in any of the groups. At the 5% levels all stools appeared the same. Several days after the hamsters began eating the 10% diets, the fecal pellets of those eating Myrj 45 became somewhat softer and less well-formed than those of the controls. This response remained the same throughout the feeding of the 15% level. However, at no time did the stools attain a fluid or even a semi-fluid character.

Macroscopic examination at autopsy revealed no evidence of pathology. Particular attention was given to the examination of the gastrointestinal and urinary tracts. Although there were some enlarged ceca in the animals on Myrj 45, no other

TABLE 3

Data relative to caloric efficiency of diets containing 15% of fat or emulsifiers

Test Material	Sex	Net Gain[1]	Food Intake[1]	EFU[2]	ECU[3]
		gm.	*gm.*		
Primex	Male	12.8	167.6	7.63	1.80
	Female	10.4	175.6	5.92	1.40
	Both	11.6	171.6	6.78	1.60
Myrj 45	Male	8.4	205.3	4.09	1.20
	Female	10.8	220.8	4.89	1.44
	Both	9.6	213.1	4.49	1.32
Esterine	Male	9.8	169.0	5.80	1.36
	Female	9.0	172.0	5.23	1.23
	Both	9.4	170.5	5.52	1.30
Myverol 18-85	Male	10.7	179.7	5.95	1.41
	Female	9.5	176.5	5.38	1.28
	Both	10.1	178.1	5.67	1.35

[1] Data from final 4 weeks of feeding.
[2] EFU = Efficiency of food utilization = grams gain per 100 grams diet.
[3] ECU = Efficiency of calorie utilization = grams gain per 100 calories. Computed from the following caloric values for the diets 423.4, 340.6, 425.8 and 421.0 calories per 100 grams, resp.

marked differences of size or condition were noted between the test and Primex groups. In fact the gross appearance, both external and internal, of these hamsters was entirely normal.

Average weights of the various organs (expressed as percentage of body weight) are shown in Table 4 for the separate and combined sexes. These data for the largest differences were examined statistically. Increased heart weights of the Esterine group and the apparent increase in thyroid weights of all the emulsifier groups were not significant. In the case of the kidneys of the Myrj 45 group, t-values indicated a significant increase in organ weight as compared to the Primex group.

The liver, kidney, duodenum, ileum, and cecum from 2 male and 2 female hamsters on each diet, and the kidneys of all the hamsters in the Myrj 45 group were examined histopathologically.

The liver of one male hamster in the Primex group showed very mild hydropic degeneration and mild congestion of the central vein and that of a female in the same group, mild central lobular degeneration of the cords. In the liver one male in the Myrj 45 group, mild focal degeneration was seen with scattered nuclear changes and mild focal round cell infiltration and in another, mild focal cord degeneration and occasional microabscesses were noted. The livers of the remaining test and control rats were normal. Kidney tissues were normal in all except one male on the Primex diet and 2 females on the Myrj 45 diet; these were only mildly congested. The absence of organic abnormalities is of interest in view of the finding that the kidneys of the Myrj 45 group were somewhat heavier than those of the other groups. One hamster of each sex on the Myrj 45 diet showed mild hyperplasia of Brunner's gland in the duodenum. The duodenum, ileum, and cecum of all other hamsters examined appeared normal histologically.

In summarizing the histopathological findings with reference to the Myrj 45 group, the consultant pathologist reported: "The livers of two of these animals (males) show hydropic changes of approximately the same degree as those of the control group. The inflammatory changes observed (microabscesses) are most likely incidental. The kidneys and the portions of the intestinal tract studied in this group do not appear to be adversely affected."

EXPERIMENT II

Procedure. Thirty-six weanling hamsters from the same source as described above were used in this test. The last 3 columns in Table 1 show the modifications in the composition of the rations in which the test components were the combination of 10% of Myrj 45 and Primex, 20% Myrj 45, and 20% Primex, respectively.

TABLE 4

Average weights of organs of hamsters fed emulsifier or fat diets

Test Material	Number and Sex	Average Body Weight[1]	Liver	Kidneys	Spleen	Heart	Adrenals	Thyroid
		gram	*per cent of body weight*					
Primex	4M	114.0	6.20	0.907	0.088	0.355	0.022	0.0046
	6F	101.6	5.98	1.001	0.141	0.351	0.015	0.0059
	10MF	107.8	6.09	0.954	0.114	0.353	0.019	0.0053
Myrj 45	6M	96.9	6.17	1.201	0.083	0.365	0.028	0.0071
	6F	102.6	5.89	1.145	0.126	0.371	0.014	0.0067
	12MF	99.8	6.03	1.173	0.104	0.368	0.021	0.0069
Esterine	4M	103.8	5.96	0.915	0.104	0.407	0.024	0.0063
	5F	97.2	5.94	1.059	0.135	0.415	0.020	0.0073
	9MF	100.5	5.95	0.987	0.120	0.411	0.022	0.0068
Myverol 18-85	5M	105.7	6.05	1.003	0.098	0.346	0.028	0.0060
	7F	95.0	5.95	1.057	0.136	0.398	0.014	0.0074
	12MF	100.4	6.00	1.030	0.117	0.372	0.021	0.0067

[1]Average weights of hamsters on day of autopsy.

The hamsters were fed the 20% Primex diet for the first week after arrival in the laboratory during the preliminary period of acclimatization. They were then distributed into 3 groups as above described and fed the experimental rations for a period of 10 weeks. Weekly body weight and food consumption records were kept, from which efficiencies of caloric utilization were calculated.

At the termination of the experiment, 4 males and 4 females from each group were sacrificed and examined at autopsy. Weights of the livers and kidneys were recorded and the liver, kidney, and gastrointestinal tracts of 2 hamsters of each sex per group were examined histopathologically.

Results. No deviations from normal behavior or appearance were observed during the 10 weeks of feeding the experimental diets containing Myrj 45 and/or Primex.

Whereas the fecal pellets of the hamsters eating the 20% Myrj 45 diet were somewhat softer and less well-formed than those of the controls, frank diarrhea was not seen in any of the groups. At no time did the stools attain even a semi-fluid character.

Growth responses are summarized in Table 5 and shown graphically in Figure 2.

TABLE 5

Growth responses of hamsters fed emulsifier, fat or a combination of them

Test Material and Level	No. of Hamsters and Sex	Average Body Weight		Net Gain at 10 wks.
		Initial	10 weeks	
		(grams)		
10% Myrj 45	7M	49.9	116.0	66.1
plus	5F	41.6	96.0	54.4
10% Primex	12MF	45.8	106.0	60.2
20% Myrj 45	4M	43.0	83.5	40.5
	7F	41.9	87.1	45.2
	11MF	42.5	85.0	42.8
20% Primex	5M	40.2	102.8	62.6
	6F	39.8	105.7	65.9
	11MF	40.0	104.3	64.2

The growth response in the 20% Myrj 45 group was somewhat less than that of the 20% Primex group. However, growth rates of the Myrj 45 plus Primex group and of the 20% Primex group were approximately the same throughout. As shown graphically in Figure 2, the weight attained by the group fed the combination of fat and emulsifier was somewhat higher than that of the group fed the fat alone, although the initial weight of the former group was also slightly higher. In any case, the difference between the net gains of the 2 groups was not significant.

Food consumption, especially in the case of the males, seemed to rise with increasing level of Myrj 45 in the diets. The caloric values for the test diets were computed (from data for Myrj 45 and Primex shown in Experiment 1) to be 395.4, 340.2, and 450.6 per 100 g. diet for the combination diet, Myrj 45, and Primex diets, respectively.

From the data in Table 6 it may be seen that the efficiency of food utilization (EFU) of the 20% Myrj 45 group was approximately half that of the Primex group, while that of the Myrj 45 plus Primex group was slighly but not significantly less than that of the Primex group.

The efficiency of caloric utilization (ECU) of the 20% Myrj 45 group was definitely diminished as compared with the Primex group. In Experiment I, the ECU of the 15% Myrj 45 group was found to be lower than that of the Primex group albeit the difference was not statistically significant. It is noteworthy, however, that in Experiment II which included the age period of 4 to 14 weeks (i.e., the period during which growth occurred and the ECU could better be estimated) the addition of 10% Myrj 45 to 10% Primex resulted in no diminution of caloric utilization.

Macroscopic examination at necropsy revealed no evidence of pathology. Except for apparent enlargement of the cecum in the 20% Myrj 45 group, the gross appearance of the hamsters in all groups was entirely normal.

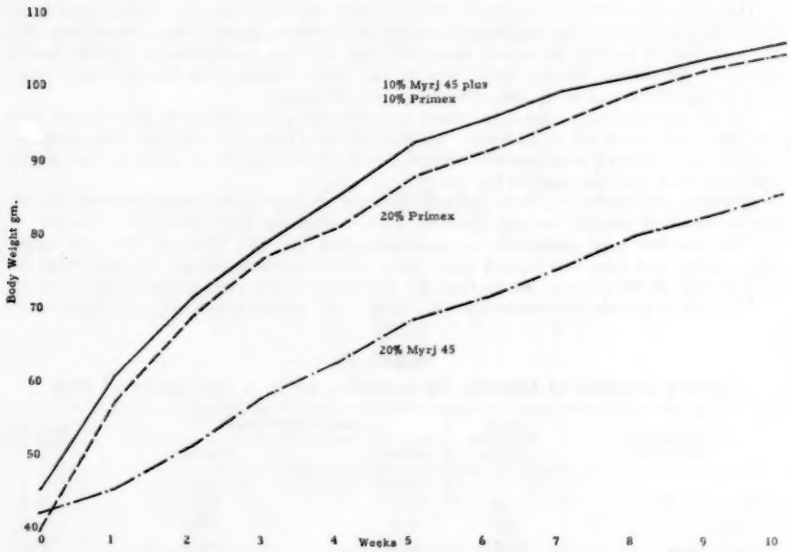

Figure 2. Growth responses of rats fed natural type diets containing 20% Primex, 20% Myrj 45 and a mixture of 10% Primex plus 10% Myrj 45.

Average weights of the livers and both kidneys (expressed as percentage of body weight) are shown in Table 7 for individual and combined sexes. The data indicate that the kidneys of the hamsters fed 20% Myrj 45 were significantly larger than those of the Primex animals. Kidneys of the Myrj 45 plus Primex group were larger, but only in the case of the females was the difference sufficient to be considered significant. As for the livers, the only significant difference was in the males on the Myrj 45 plus Primex diet whose livers were heavier than those in the other groups. Tissues of 2 hamsters of each sex in each group were examined histopathologically. In the group fed Primex the livers of all 4 rats showed changes varying from slight to moderately severe subacute or acute toxic hepatic cord changes, one showing early portal cirrhosis.

TABLE 6

Caloric efficiency of diets containing emulsifier, fat, or a combination of them

Test Material and Level	No. of Hamsters and Sex	Net Gain[1]	Food Intake[1]	EFU[2]	ECU[3]
		gm.	*gm.*		
10% Myrj 45	7M	66.1	473.6	14.0	3.54
plus	5F	54.4	437.6	12.4	3.14
10% Primex	12MF	60.2	455.6	13.2	3.34
20% Myrj 45	4M	40.5	513.7	7.9	2.32
	7F	45.2	541.5	8.3	2.44
	11MF	42.8	527.6	8.1	2.38
20% Primex	5M	62.6	407.8	15.4	3.42
	6F	65.9	432.7	15.2	3.37
	11MF	64.2	420.8	15.3	3.40

[1] Average data for 10-week period.
[2] EFU = Efficiency of food utilization = grams gain per 100 grams eaten.
[3] ECU = Efficeincy of calorie utilization = grams gain per 100 calories eaten: the caloric values of the diets were computed as 395.4, 340.2 and 450.6 calories per 100 grams, respectively.

TABLE 7

Average weights of organs of hamsters[1]

Test Material and Level	Sex	Average Body Weight[2]	Liver	Kidneys
		gm.	*per cent of body weight*	
10% Myrj 45 plus 10% Primex	Male	124.3	7.09	1.016
	Female	103.8	6.27	1.198
	Both	114.1	6.68	1.107
20% Myrj 45	Male	88.8	6.06	1.214
	Female	90.5	5.91	1.286
	Both	89.7	5.99	1.250
20% Primex	Male	114.3	5.81	0.915
	Female	122.5	6.20	0.982
	Both	118.4	6.01	0.949

[1] Averages of four hamsters of each sex per group.
[2] Average weight of hamsters on day of autopsy.

The most advanced lesion was in a female in which a moderately severe "yellow atrophy" or diffuse necrosis of recent origin was observed. The histopathologist's report states that "All of these changes appear to be compatible with recovery on cessation of experimental conditions."

In the group fed the combination of Myrj 45 plus Primex, the livers of one female and of two of the males showed changes associated with occasional mild hydropic disarrangement with lobular swelling and a suggestion of regeneration or a recovery phase of a cytoplasmic irritant nature. The liver of the second female was normal. These effects were considered "reversible and transient." In the group fed Myrj 45 alone the liver of one male showed mild changes similar to those in the preceding group (Myrj 45 plus Primex); the livers of the other 3 hamsters appeared normal. The kidneys in all groups appeared to be normal. This finding is of interest in view of the fact that the kidneys in the Myrj 45 group were slightly heavier in relation to body weight than those of the other groups. However, it may be pointed out that increased organ weights, even in the absence of microscopic pathology, are regarded by some investigators as indicative of a stress condition "predictive of injury to be expected at higher intake levels (but cf. the levels here employed) or perhaps after a longer period of treatment" *(24)*.

DISCUSSION

The studies presented in this report on the effect of abnormally high levels of polyoxyethylene (8) monostearate and monoglycerides on hamster physiology differ from those reported elsewhere by the employment of natural rather than "purified" diets, including supplementation with all known growth factors, in order to insure satisfying the nutritional and physiological dietary requirements for this species of animal.

At the 5% dietary level none of the test materials caused retarded growth. At the 10 and 15% levels, however, though there were no significant differences in responses to the emulsifier diets (Myrj 45, Esterine, and Myverol 18–85), growth was somewhat less than on the Primex diet. However, when weight gains were related to the caloric values of the supplemented diets, the small differences in the efficiency of utilization observed between the Primex and the emulsifier diets were found not to be significant. These studies thereby confirm and extend other reports. Krehl *et al. (12)* observed no growth retardation upon feeding polyoxyethylene (8) monostearate to rats and cats in a diet containing Ruffex and dex-

trinized starch although increased food intake resulted from the lowered caloric density. Similar results were obtained with rats by Graham and Grice *(7, 8)* on diets containing flour plus Alphacel and by Preston *et al. (20)* with children, in both cases with natural-type diets. Not only did Chow *et al. (2)* observe no growth retardation by feeding 5 or 15% Myrj 52 (polyoxyethylene (40) stearate) in a soybean meal diet to rats, but they further demonstrated that the addition of 8% Celluflour to a 10% Myrj 52 diet containing soybean meal, a natural bulk producer, resulted in markedly enhanced growth and no increase in food intake despite the reduction in caloric density.

Although Poling *et al. (4, 19)* reported growth retardation in rats fed a purified diet containing 25% of a polyoxyethylene stearate similar to Myrj 45 for 21 weeks, they found it necessary for unexplained reasons, to raise the protein level of their diets from 18 to 24% at 8 weeks. Subsequently in this experiment the gains were not significantly less than those of the lard-fed controls.

Schweigert *et al. (23)* employed a synthetic diet containing 3% Celluflour, and reported retarded growth and poor food utilization of weanling hamsters. The efficiency of food utilization in the 15% Myrj 45 group was not significantly different from that in the 5% group, hence it would appear that impalatability rather than toxicity may have been the significant factor. It is noteworthy that the group which ingested 7.5% lard plus 7.5% Celluflour, grew more rapidly that the group on 15% lard despite the higher caloric density of the latter diet. This observation supports the well-recognized need of hamsters for roughage and suggests that the requirement may be accentuated in synthetic high fat diets where the carbohydrate is supplied by sucrose rather than by polysaccharides ranging in complexity from starch to cellulose. (The natural diet employed in the studies here reported contained approximately 4% intrinsic fat and 2.5% crude fiber.)

Both the food intake data and post-mortem findings in Poling's growth studies with hamsters on purified diets *(19)* strongly suggest that their animals at the 25% polyoxyethylene stearate level rejected the diet and died of starvation. Deaths at 7 or 8 months occurred not only at the 15% experimental group but in the 15% lard group as well, and in neither case should be regarded as of dietary origin.

Inanition due to non-acceptance of the diet appears also to account for the response of their hamsters fed "bread" diets *(19)*, whereby weight losses occurred initially with increasing levels of Myrj 45 but normal rates of gain were resumed at about the 4th week. Computations based on these authors' data reveal that increasing the level of lard resulted in increased efficiency of caloric utilization; however, caloric efficiency, while not increased was not depressed at the higher levels of Myrj 45. This is of particular interest from the toxicological standpoint inasmuch as the partial ester emulsifiers are not intended for use as replacements for lard or any other food fat and at the normal use concentrations have no effect either on caloric density, palatability, or bulk of the diet.

No gross pathology was observed in our hamster experiments on either emulsifier or Primex diets, though in the Myrj 45 group, the ceca and

kidneys were somewhat enlarged. Microscopically, all organs including the gastrointestinal and urinary tracts were normal. No diarrhea was observed in these animals nor was there any indication of hematuria. Absence of pathology after feeding Myrj 45 at abnormally high levels for prolonged periods of time confirmed studies by others with rats, cats, and humans *(1, 2, 3, 7, 8, 12, 13, 14, 20)*.

Eagle and Poling *(4)* present their pathological findings in terms of per cent, a procedure that can be somewhat misleading when groups are as small (7 to 10) as those examined. Critical reexamination of their data indicate either the undesirability of the high level of lard in the diet, a poor basal diet, or latent initial pathology in their experimental animals. In any case, the pathological findings in the control rats, cast considerable doubt on the authors' implication that similar findings in the rats fed polyesters are of toxic origin.

It is noteworthy that many of the pathological findings observed in their hamsters are characteristic of vitamin E deficiency. These include renal calcification *(2, 6)*, gastric ulcers *(11)*, testicular degeneration *(6)*, and especially the deposition of a yellow brown pigment. Mason and Emmel are reported to have found "small globules of pigment . . . in the uterine muscle fibers, ovary, interstitial cells of the testis, lymph nodes, spleen, fat, macrophages of the liver, kidney" and other organs *(6)*. Although Eagle and Poling found iron in pigmented tissue, the possibility is not precluded that some of this pigmentation may have been associated with vitamin E deficiency. Inspection of the purified rat diets reveals that tocopherols were not added, the only source of vitamin E being the vitamin A and D carrier oil. Although wheat germ oil was added to the "bread" rations fed to the hamsters, a chronic vitamin E deficiency may account at least partially for the persistent pathology in the control animals. Parenthetically it may be noted that hamsters, which normally mature as early as 28 days of age and have a short gestation period, are extremely sensitive to vitamin E shortage *(10, 17, 22)*. Nevertheless, Eagle and Poling refer to work by Wissler *et al. (4, 26)* in which the hemosiderosis is attributed to increased iron absorption via the cecum. Since there is no organ in the human equivalent to the cecum of the rat or hamster in structure and in function, the occurrence of hemosiderosis via the cecum in these animals has no relation to human physiology, and therefore is probably of no significance.

It is well known that hamster urine is normally thick with phosphatic sediment. Under conditions of dehydration and hemoconcentration (cf. the observed increase in water intake) resulting from diarrhea, it would be expected that urinary precipitation would be augmented and the tendency for calculus formation with attendant inflammation of the urinary tract would be increased. Moreover, to the extent that calcium soaps are formed in the intestinal lumen thereby diverting phosphate from the intestinal to the urinary tract, this would be exaggerated on diets containing high levels of fatty acid *(9)*. These points would appear to be significant in relation to Eagle and Poling's *(4)* findings as to incidence of renal concretion in both the lard controls (viz. 3 out of 9) and the Myrj 45 rats (viz. 2 out of 5).

Culver *et al. (3)* have shown that in the human polyols are cleared rapidly via the kidney. Hence the finding of levels of 0.6 to 2.3 grams per cent polyoxyethylene glycol in the blood of Poling's rats is very surprising. It is suggested that the dehydration and urinary tract inflammation are interrelated with the high polyol blood levels they observed.

The contrast between the relatively innocuous effects of high level feeding of Myrj 45 observed in our laboratories *(15, 16)* and elsewhere and the findings of Schweigert, and Poling and their associates, is in no small measure related to the reaction of hamsters to natural vs. synthetic diets. The fact that the nutritional requirements of this species have not been fully elucidated nor their physiological idiosyncrasies well understood is given emphasis by the recent report of Ershoff *(5)* showing that hamsters obtained from two of three different suppliers failed to survive 5 weeks on a purified ration composed of casein plus sucrose or glucose whereas hamsters from the third source thrived. On the other hand, hamsters from all 3 sources survived and grew normally when fed a natural ration, or a purified ration in which cornstarch replaced the sugar or to which aureomycin or alfalfa were added.

SUMMARY

Two experiments are reported in which a hydrogenated vegetable oil (Primex), polyoxyethylene (8) monostearate (Myrj 45), a mixture of meat and vegetable monoglycerides and diglycerides (Esterine), and cottonseed oil monoglycerides (Myverol 18–85) were fed to hamsters at varying levels from 5 to 20% of a diet composed of natural ingredients and simulating the American dietary, for varying periods up to 10 weeks. No abnormalities in behavior or physical appearance were observed in any of the hamsters at any time during the feeding periods. Diarrhea was not observed in any of the groups and no mortality was attributable to the dietary regimes. No significant differences in weight gains were noted among the three groups fed Myrj 45, Esterine, or Myverol 18–85 although all three groups grew at a somewhat retarded rate compared with the group fed Primex. Although with Myrj 45 at the 20% level growth was somewhat retarded, the combination of 10% Primex and 10% Myrj 45 resulted in a rate of gain and efficiency of calorie utilization equal to that of the 20% Primex group. The high voluntary food intake of the Myrj 45 group was related to the low caloric density of that diet. The gross appearance of the animals, both external and internal, was normal although the ceca and kidneys of the Myrj 45 group were somewhat enlarged. Microscopic studies demonstrated the absence of pathology in the following organs: liver, kidney, duodenum, ileum, and cecum.

The lack of harmful effects upon feeding polyoxyethylene (8) monostearate (Myrj 45) to hamsters at dietary levels as high as 20% confirms the conclusion reached by other investigators working with man as well as with experimental animals, that no hazard may be expected from the proposed use of this emulsifier to the extent of a few tenths of 1% in the human diet.

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LITERATURE CITED

1. Allison, J. B., Rosenthal, H. L., and Mills, A. H. Effects of nonionic surface active agents on the growth of animals. *Fed. Proc.*, **11**, 435 (1952).
2. Chow, B. F., Burnett, J. M., Ling, C. T., and Barrows, L. Effects of basal diets on the response of rats to certain dietary non-ionic surface-active agents. *J. Nutrition*, **49**, 563-578 (1953).
3. Culver, P. J., Wilcox, C. S., Jones, C. M., Rose, Jr., R. S. Intermediary metabolism of certain polyoxyethylene derivatives in man. I. Recovery of the polyoxyethylene moiety from urine and feces following ingestion of polyoxyethylene (20) sorbitan monooleate and of polyoxyethylene (40) monostearate. *J. Pharmacol. Exp. Therap.*, **103**, 377-381 (1951).
4. Eagle, E., and Poling, C. E. The oral toxicity and pathology of polyoxyethylene derivatives in rats and hamsters. *Food Research*, **21**, 348-361 (1956).
5. Ershoff, B. H. Beneficial effects of alfalfa, aureomycin and cornstarch on the growth and survival of hamsters fed highly purified rations. *J. Nutrition*, **95**, 579-585 (1956).
6. Follis, R. H., Jr. *The Pathology of Nutritional Disease.* 1948, Charles C. Thomas, Publisher, Springfield, Illinois.
7. Graham, W. D., and Grice, H. C. Chronic toxicity of bread additives to rats. II. *J. Pharm. and Pharm.*, **7**, 126-134 (1955).
8. Graham, W. D., Teed, H., and Grice, H. C. Chronic toxicity of bread additives to rats. *J. Pharm. Pharmacol.*, **6**, 534-545 (1954).
9. Gyorgy, P. (Personal communication.)
10. Hamilton, J. W., and Hogan, A. G. Nutritional requirements of the Syrian hamster. *J. Nutrition*, **27**, 213-224 (1944).
11. Harris, P. L., Hove, E. L., Mellot, M., and Hickman, K. Dietary production of gastric ulcers in rats and prevention by tocopherol administration. *Proc. Soc. Exp. Biol. Med.*, **64**, 273-277 (1947).
12. Krehl, W. A., Cowgill, G. R., and Whedon, A. D. Non-deleterious effects of polyoxyethylene esters in the nutrition of rats and cats. *J. Nutrition*, **55**, 35-61 (1955).
13. Kruesi, O. R., and Van Itallie, T. B. Effect of polyoxyethylene-8-monostearate (Myrj 45) on liver function in patients convalescing from hepatic disorders. *Food Research*, **21**, 565-569 (1956).
14. Oler, W. M., and Craemer, V. C. Effects of two commercial emulsifiers, Myrj 45 and Myrj 52, on gastric acidity and gastrointestinal motility of human subjects. *Gastroenterology*, **28**, 281-287 (1955).
15. Oser, B. L., and Oser, M. Nutritional studies on rats on diets containing high levels of partial ester emulsifiers. I. General plan and procedures: growth and food utilization. *J. Nutrition*, **60**, 367-390 (1956).
16. Oser, B. L., and Oser, M. Nutritional studies on rats on diets containing high levels of partial ester emulsifiers. III. Clinical and metabolic observations. *J. Nutrition*, **61**, 149-166 (1957).
17. Pappenheimer, A. W. *On Certain Aspects of Vitamin E Deficiency.* 1948, Charles C. Thomas, Springfield, Ill.
18. Poiley, S. M. Breeding and care of the Syrian hamster. *The Care and Breeding of Laboratory Animals.* 1950, 118-152, John Wiley and Sons, Inc., New York.
19. Poling, C. E., Eagle, E., and Rice, E. E. Effects of feeding polyoxyethylene preparations to rats and hamsters. *Food Research*, **21**, 337-347 (1956).
20. Preston III, E., Hunt, A. D., Jr., McNair, S. T. F., and Spur, B. Short-term feeding studies in infants and children with certain surface-active agents used in food technology: Absence of evidence of toxic effects. *J. Clin. Nutrition*, **1**, 539-550 (1953).
21. Schroeder, L. J., Hiratzka, T., and Smith, A. H. Torula yeast and liver necrosis. *Fed. Proc.*, **15**, 572 (1956).
22. Schweigert, B. S., Nutritional requirements of the cotton rat and hamster. *Vitamins and Hormones*, **VI**, 55-67 (1948).
23. Schweigert, B. S., McBride, B. H., and Carlson, A. J. Effect of feeding polyoxyethylene monostearates on growth rate and gross pathology of weanling hamsters. *Proc. Soc. Exp. Biol. Med.*, **73**, 427-432 (1950).

24. SMYTH, H. S., JR., WEIL, C. S., ADAMS, E. M., AND HOLLINGSWORTH, R. L. Efficiency of criteria of stress of toxicological tests. *Arch. Ind. Hyg. Occupat. Med.*, **6**, 32-36 (1952).
25. U. S. Department of Health, Education, and Welfare, Ice Cream Hearings, Docket No. FDC-34(a) (1952).
26. WISSLER, R. W., BETHARD, W. F., BARKER, P., AND MORI, H. D. Effects of polyoxyethylene sorbitan monolaurate (Tween 20) upon gastrointestinal iron absorption in hamsters. *Proc. Soc. Exp. Biol. Med.*, **86**, 170 (1954).
27. WANG, H., MCBRIDE, B. H., AND SCHWEIGERT, B. S. Histoiogical manifestations of feeding polyoxyethylene monostearates to weanling hamsters. *Proc. Soc. Exp. Biol. Med.*, **75**, 342-348 (1950).
28. WORDEN, A. N. *The Care and Management of Laboratory Animals.* 1947, 196-202, Williams and Wilkins Company, Baltimore, Md.

OXIDATIVE FAT RANCIDITY IN FOOD PRODUCTS. II. WALNUTS AND OTHER NUT MEATS[a]

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Increased marketing of shelled nut meats in transparent flexible plastic films and similar containers has emphasized the problems of stabilization of the nut meats against oxidative fat rancidity *(20)*. Methods of stabilization would be ideally based on a thorough knowledge of the inherent factors which make the nut meats susceptible or resistant to oxidative fat rancidity. Knowledge of these factors is largely lacking in the case of English Walnuts *(Juglans regia)* and most other nut meats.

The walnut is known to contain highly unsaturated oil which might oxidize readily when the pellicle is broken and oil is oriented on the nut meat surface. However, there have been no studies to define quantitatively the importance of exposed nut meat surface. Besides this surface-catalyzed autoxidation of the oil, lipid peroxidation biocatalysts may be present in the walnut, and these may greatly accelerate rancidity. Since free fatty acids oxidize more readily than triglycerides, much importance has been ascribed to the presence of a lipase and the resulting development of free fatty acids in walnuts. Although free fatty acids do develop during storage under conditions suggesting lipase catalysts *(19)*, the activity of the walnut lipase has never been measured *in vitro*. Use of this enzyme in simple assay systems is a prerequisite to studies of its catalytic properties. Since tocopherols are the principal biological antioxidants which inhibit oxidation of the unsaturated lipids, the tocopherol content of walnuts should be determined.

Besides the need for defining the factors which influence oxidative fat rancidity in the walnut, there is a need for better objective methods for measuring this oxidative rancidity. Peroxide values are used industrially and were used in some previous studies *(19)*. However, our present knowledge of oxidative fat rancidity shows carbonyl compounds to be responsible for rancid flavors and odors. Methods based on the 2,4-dinitrophenylhydrazine or the thiobarbituric acid reactions with carbonyls and other products of oxidative fat rancidity have been found applicable to a wide variety of foods including fats *(12)*, pork *(25)*, cereal products *(6)*, and dried milk *(23)*. Studies of the applicability of these methods for objective measure of oxidative fat rancidity in walnuts are needed.

It was the purpose of this investigation to define some of the factors influencing the stability of walnuts against oxidative fat rancidity and to determine an objective method for measuring rancidity.

EXPERIMENTAL

The walnuts, almonds, filberts and peanuts used in this research were obtained

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from the Diamond Walnut Growers, Inc., California Almond Growers Exchange, Northwest Nut Growers, and Southern Utilization Research Branch, respectively. The pecans and black walnuts were obtained from local sources.

Oxygen absorption. Uniformly sized pieces of walnut meats were obtained from the Diamond Walnut Growers, Inc. These walnut meat pieces averaged .026 g. per piece and had an average area of 16 cm.^{2}/g. The water content determined by xylene distillation was 2.7% and by vacuum oven was 2.5%. Fat determined by ether extraction was 65.4%. For measurement of oxygen absorption, 50 g. of walnut meat pieces were placed in a 125 ml. Warburg flask and allowed to oxidize at 37° C. in an atmosphere of oxygen. For studies of the oxidation of walnut oil, oil was pressed from the walnut pieces in a Carver press. Six ml. of oil were layered out in the bottom of each 125 ml. Warburg flask, giving an exposed surface of 1.5 cm.^{2}/g. The oil was allowed to oxidize at 37° C. in an atmosphere of oxygen. The oxygen absorption of nut meat pieces and oil was run in triplicate.

Oxidative catalysts. The protein portion of nut meats was separated by first extracting the lipids and water with 10 ml. butanol/g. ground nut meats at 0° C. for 1 hr. The filtered protein was then extracted with 5 ml. diethyl ether/g. nut meat and dried in vacuum.

The nut meat protein was assayed for oxidative catalysts under conditions suitable for the majority of lipoxidase enzymes *(22)* and hematin compounds *(24)*. One ml. of the extract of 1 g. protein/10 ml. phosphate buffer was shaken with 4 ml. 0.02 M ammonium linoleate in 0.01 M phosphate buffer, pH 7, in a Warburg manometric apparatus at 20° C.

Lipase. The first method used for measuring lipase activity was a direct measure of free fatty acids liberated from triglyceride emulsions in the presence of a variety of walnut extracts. Subsequently, the more sensitive method of Luchinger *et al. (18)* was used to measure the glycerol liberated when the lipase of walnuts catalyzed the hydrolysis of monoglycerides. For this assay walnut extract was prepared by mixing 0.5 g. of petroleum ether defatted walnut powder with 10 ml. distilled water for 10 minutes. Following centrifugation, the clear supernatant was used as the enzyme source. The monoglyceride emulsion consisted of 0.5 g. of glycerol-free monoglyceride of cotton seed oil homogenized with 100 ml. distilled water. For assay, 1 ml. of monoglyceride emulsion, plus 2 ml. phosphate buffer, pH 7.4, were shaken in a water bath at 30° C., and 1 ml. of walnut extract was added after temperature equilibration. At time intervals, 1 ml. of the reaction mixture was pipetted out, and the reaction was stopped by the addition of 0.1 ml. of an inactivating solution consisting of 5 g. calcium chloride and 10 g. trichloroacetic acid in 85 ml. of water. Glycerol produced by the enzyme reaction was measured colorimetrically *(16)* in a Klett-Summerson photocolorimeter using a 450 mμ filter. Controls were used to correct for the small amount of hydrolysis which occurred when monoglyceride was incubated in the presence of water or heat denatured walnut extract instead of undenatured walnut extract.

Tocopherol content. One gram of ground nut meat was ground with benzene in a mortar and made up to 25 ml. volume and allowed to stand 0.5 hr. Following centrifugation, an aliquot was reacted with 1 ml. of 0.5% α,α′-bipyridyl in glacial acetic acid and 1 ml. of 0.25% ferric chloride in acetic acid. Following the addition of ferric chloride, absorbance at 515 mμ was measured in an Evelyn colorimeter at frequent time intervals. Pure α-tocopherol was used as the standard.

In the method used *(7)*, absorbance at 515 mμ, due to the tocopherols, reaches a maximum within 2 min. after the addition of ferric chloride. When, in addition to tocopherols, less strongly reducing substances are also present, the characteristic sharp increase in absorbance is followed by a slower approach to a maximum. In the latter case it is assumed that calculated values based on absorbance at 2 min. will represent all of the tocopherols with a minimal contribution by the other reducing substances present.

Thiobarbituric acid reaction. Ten g. of ground nut meats were placed in an 800 ml. Kjeldahl flask, and 125 ml. of 0.025 N hydrochloric acid were added. The flask was heated gently, and the first 50 ml. of distillate were collected in a volumetric flask. Ten ml. of the distillate were added to 2 ml. of the thiobarbituric acid reagent and made to 100 ml. with glacial acetic acid. The thiobarbituric reagent consisted of 0.67 g. thiobarbituric acid, 75 ml. of glacial acetic acid, and 2 ml. hydrochloric acid heated

on a steam bath to facilitate solution. The distillate and the thiobarbituric acid reagent in acetic acid were heated at 100° C. for 35 min. The reaction was cooled to room temperature and the color developed was measured with a Klett-Summerson colorimeter using a 540 mμ filter.

Organoleptic scoring. The experimental design consisted of a balanced complete block of 10 blocks, 3 samples per block, 6 replications per sample and the appearance of each sample with every other sample 3 times. An eleven point scoring scale was used with 0 equal to no rancidity, 1, 2, 3, slight rancidity, 4, 5, 6, moderate rancidity, and 7, 8, 9, 10 extreme rancidity. Tasting was done under red illumination to overcome color differences. The panel consisted of 7 people who were selected from a group of 15 for their ability to detect and score standard walnut samples having varying degrees of rancidity.

RESULTS AND DISCUSSION

Oxygen absorption. The oxygen absorption of walnut pieces and the equivalent amount of walnut oil are shown in Figure 1. The walnut oil had an induction period of 200 hours during which no oxygen absorption took place. This induction period was followed by a rapid autocatalytic oxygen absorption which is typical of vegetable oils containing tocopherol. The liquid condition of the oil allowed the intermixing of reaction components so that the tocopherol and other antioxidants present afforded protection against oxidation for 200 hrs. Following this induction period the free mixture of reaction components allowed rapid initiation and propagation of the oxidation. In contrast to this behavior, the walnut pieces showed an immediate oxygen absorption, followed by a constant rate of oxidation lasting over 1000 hrs., and then a gradual decrease. This behavior pattern can largely be attributed to the fact that the lipid in the walnut pieces was immobilized as a surface layer. The exposed surface (amounting to over 10 times that of the oil equivalent to the nut meats) allowed immediate reaction between the highly unsaturated lipids

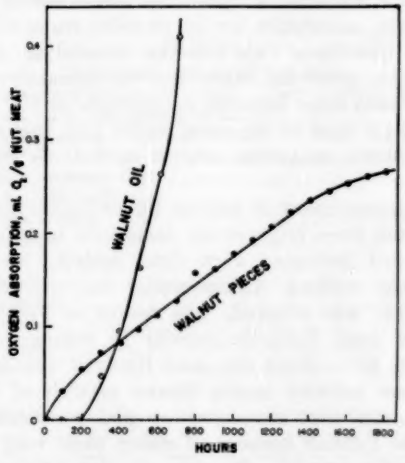

Figure 1. Comparison of the oxidation of walnut oil when present in kernel pieces and in the liquid phase.

and the oxygen. The large reaction rate at the beginning of the reaction might be attributed to surface catalyzed autoxidation and to the relatively large amount of unsaturated lipids free to oxidize. During the first portion of the reaction there are increasing amounts of lipid oxidation products and decreasing amounts of lipid free to oxidize. During the long period of constant oxidation rate, the amount of lipid oxidation products and the rate of chain propagation would probably be high; the rate of chain initiation must equal the rate of chain termination, and limiting factor is probably unreacted unsaturated lipid. The accumulation of lipid oxidation products such as lipid oxidation polymers, probably accounts for the decrease of the reaction rate at about 1500 hrs. Results with ground walnuts are interesting but will not be presented in detail. Ground walnuts showed a pattern of oxidation which is intermediate between walnut pieces and free oil. Duplicate samples showed an induction period of approximately 20 hrs. followed by a period of slow oxygen absorption up to 120 hrs. and then a rapid steady state reaction up to 300 hrs. when the reaction was terminated. This rapid steady state reaction was three times faster than that shown in Figure 1 for the walnut pieces.

The oxygen absorption of walnut pieces shown in Figure 1 indicates that the lipid antioxidants afford little protection against surface catalyzed autoxidation, especially when the protective walnut pellicle is broken or lost. It is most significant that there was no induction period but rather a rapid oxidation which continued undiminished until the walnut pieces became rancid and the oxidation reaction slowed down. This experimental evidence indicates that commercial storage of walnut pieces in the presence of any oxygen must involve lipid oxidation, a progression toward oxidative fat rancidity, and hence, some reduction in quality.

Oxidative catalysts. The protein of almonds, filberts, pecans, and black walnuts gave no catalytic oxidation of linoleate. Under these assay conditions peanuts and walnuts catalyzed the oxidation of linoleate at rates of 1.8 and 0.7 ml. O_2 absorption/hr./g. protein, respectively. Peanuts are known to contain lipoxidase *(22)* but the biocatalyst in walnuts has not been identified. This assay indicates that the biocatalyst in walnuts may exist in small amounts since hematin compounds at 10^{-6} M or a lipoxidase content equal to 0.4% that of soybeans would give this result. The identification of the linoleate oxidation catalyst in walnuts will require further research.

Lipase. The measurement of walnut lipase by the titration of the free fatty acids liberated from triglyceride emulsions to which various walnut extracts were added indicated very little activity and the need for a more sensitive assay method. Consequently the very sensitive method of Luchinger *et al. (18)* was adopted. The results in Table 1 show that walnuts contain only weak lipolytic activity in comparison to many other oil seeds. Walnuts have about the same lipolytic activity as wheat. This low order of lipase activity makes kinetic studies of these lipases very difficult and indicates that concentration and purification of the lipases of walnuts may be difficult because of either their very low concentration or their low specific activity. Previous studies *(19)* which indicate the importance of lipases in the oxidative deterioration of walnuts must

TABLE 1

Lipase activity of walnuts

Walnut variety used as enzyme source	% glycerol liberated from monoglyceride	
	90 min.	165 min.
Lg. Eurekas (Chico)	0.72	0.90
Lg. Eurekas (Yuba)	0.64	0.72
Lg. Franquettes	0.72	0.68
Buds	0.64	0.98

be reconciled with this low order of lipase activity. Previous estimates of lipase activity were based on free fatty acid liberation after long storage periods of 1 to 3 months and unusually high water contents. Furthermore, direct attempts to measure lipase *in vitro* were previously unsuccessful.

Tocopherols. Tocopherol contents of the nuts assayed are presented in Table 2. In the assays of walnut, peanut and pecan extracts, maximum absorbance values were not reached until 10 to 15 min. had elapsed. Tocopherol contents for these nuts, calculated on the basis of absorbance values at 2 min., may be accepted as closely approaching true values. In the benzene extracts of almond and filbert kernels maximum absorbance was recorded within the 2 min. interval. Thus, calculated tocopherol values for these two nuts may be viewed with greater confidence.

Table 3 gives averages of literature values for total tocopherols of the five nuts. Also presented are iodine values and per cent of unsaturated glycerides of the nut oils. Literature values for tocopherols in nuts are generally given as mg./100 g. of oil. In order to facilitate comparisons on a whole-kernel basis, average oil contents of the nuts have been employed in calculating the tocopherol values of nut kernels. Data for tocopherols in English walnuts appear to be absent from the literature. Only one reference was found for the tocopherol content of almonds.

TABLE 2

Tocopherol content of nut meats

Nut meats, variety and condition or treatment	Total tocopherols mg./100 g.
Walnut, Lg. Eurekas, fresh	25–30
Walnut, Lg. Eurekas, peeled meats (87%)	17
Walnut, Lg. Eurekas, pellicle (13%)	29–31
Walnut, Lg. Franquettes, fresh	20–25
Walnut, Lg. Mayettes, fresh	24
Almond, Non-parell, fresh	35.0
Almond, Non-parell, blanched	27.0
Almond, Non-parell, roasted	7.4
Almond, Non-parell, rancid	26.5
Almond, Mission, fresh	33.5
Filbert, fresh	28.0
Pecan, fresh	20.0
Peanut, Spanish, fresh	19.0

TABLE 3

Average literature values for the composition of nut meats

Nut meats	Iodine value of oil	Unsaturated glycerides			Total tocopherols	References
		Monoenoic	Dienoic	Trienoic		
		%	%	%	mg./100 g. nut meat	
Walnut	147	16	63	6	....	*9, 11, 15, 21, 26, 27*
Peanut	95	50	30	0	21	*1, 8, 17*
Filbert	91	81	11	0	28	*2, 10, 15, 17, 26*
Pecan	102	72	21	0	32	*1, 3, 4, 13, 15, 17*
Almond	104	77	19	0	22	*5, 9, 13, 15*

Table 3 indicates much higher per cent of unsaturation in the walnut lipids than in the glycerides of the other nuts listed. This is reflected in the approximately 50% higher iodine value of walnut oil. A high correlation has been reported between the per cent of linoleic acid and tocopherol content in fats and oils *(13)*. However, walnut oil appears to be an exception to this rule. The contrast between the high unsaturation of walnut oil and the relatively low tocopherol content of walnuts may provide an explanation for the low storage-stability of walnut oil and broken walnut kernels.

The thiobarbituric acid test for rancidity. In seeking an objective chemical method for quantitative determination of oxidative fat rancidity in walnuts, this research was directed toward the measurement of carbonyl compounds. Exploratory research showed that ground walnuts held in refrigeration under nitrogen, stored at 90°F. for 7 months and stored at 130° F. for 7 months had carbonyl concentrations of 2×10^{-3} M, 4×10^{-3} M and 10×10^{-3} M respectively. Further evaluation of the 2,4-dinitrophenylhydrazine and the thiobarbituric acid reactions as measures of the products of oxidative fat rancidity showed that a modification of the thiobarbituric acid method offered the best means of measuring rancidity in walnuts. A search of various methods for separating from walnuts the carbonyl compounds and other products of rancidity which give reaction with thiobarbituric acid reagent showed that steam distillation was applicable. Consequently a simplified distillation using the Kjeldahl assembly was adopted. Results in Table 4 show that this method of thiobarbituric acid assay gives increasing absorbance with storage of the ground walnuts

TABLE 4

Thiobarbituric acid assay and organoleptic scores as measures of walnut rancidity

Storage at 95°F., weeks	Absorbance at 540 mμ	Organoleptic scores
0	.092	1.6
1	.104	2.4
2	.122	3.5
3	.190	3.5
4	.246	4.3
5	.348	
6	.390	
7	.512	

and correlates well with organoleptic scores. The color formed in the thiobarbituric acid reaction increases as a function of time to 7 weeks at 95° F., at which time the walnuts are very rancid and out of the rancidity range found in practice. The thiobarbituric acid reaction is simple to run, the absorbance of color increases with increasing storage, and hence, oxidative deterioration and the absorbance of color correlate well with organoleptic scores in the important region—slightly rancid to moderately rancid. Therefore, this method appears very useful for evaluating oxidative deterioration in walnuts and walnut products.

Using exactly the same method, its applicability to almonds, pecans and filberts was evaluated. Ground almonds held for 6 weeks at 95° F. developed an off-flavor in 4 weeks and became rancid in 5 weeks but showed no increased color in the thiobarbituric acid reaction. Ground pecans, held for 5 weeks, were slightly rancid after 3 weeks and were definitely rancid on the 4th week, but gave no color increase in the thiobarbituric acid reaction. Ground filberts, held for 5 weeks, became off-flavored in 3 weeks and definitely rancid in the 4th week, but they also showed no color increase in the thiobarbituric acid reaction. The applicability of this modified thiobarbituric acid reaction as a measure of oxidative fat rancidity in walnuts but not in almonds, pecans and filberts may be ascribed to the fact that trienoic fatty acids occur in walnuts but not in almonds, pecans and filberts (Table 3). The thiobarbituric acid reaction is primarily a measure of the oxidation products of linolenic acid *(14)*.

SUMMARY

Investigations were conducted in order to define some of the factors influencing the storage stability of English walnuts towards oxidative rancidity. Measurement of oxygen uptake by walnut oil showed a 200-hour induction period followed by rapid autoxidation. Walnut pieces began to oxidize immediately; a steady rate of oxygen uptake lasting 1000 hours was followed by a gradual decrease. The significance of the oxidation rates is discussed, and the importance of excluding oxygen from commercially stored walnut pieces is pointed out. Walnut protein extracts were shown to contain an unidentified linoleate-oxidation biocatalyst in low concentration. *In vitro* assay of walnut lipase indicated that this enzyme either possesses low specific activity or is present in limited concentration. The tocopherol content of walnuts was found to be of the same order (20-30 mg./100 g.) as that found in peanuts, pecans, almonds and filberts. The literature indicates that the oil of walnuts contains 50% more unsaturation than does the oil of these other nuts, possibly explaining the low storage stability of walnut pieces exposed to oxygen.

The thiobarbituric acid test (following steam distillation of volatile rancidity products) was found to correlate well with storage time and organoleptic scores of walnuts but not of almonds, pecans and filberts. This test provides a simple and reliable objective means for measuring the oxidative deterioration of walnuts.

Acknowledgments

The authors express their sincere appreciation to Mr. Donald M. Swarthout, Director of Research, Diamond Walnut Growers, Inc., for help and suggestions throughout the course of this study.

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LITERATURE CITED

1. Bailey, A. E. *Industrial Oil and Fat Products.* 1945, Interscience Publishers Inc., New York.
2. Bertram, S. H. Hazelnut oil. *Ole, Fette, Wachse, Seife, Kosmetik,* **1936**, No. 14, 2; through *Chem. Abstr.* **31**, 897.
3. Bickford, W. G., Mann, G. E., and Markley, K. S. Possibilities of peanut, pecan and safflower seed oils as supplements for olive oil. *Oil and Soap,* **20**, 85 (1943).
4. Boone, P. D. Chemical constituents of pecan oil. *Ind. Eng. Chem.,* **16**, 54 (1924).
5. Bush, W. A., and Lasher, E. A. Iodine number of expressed almond oil. *Ind. Eng. Chem.,* **33**, 1275 (1941).
6. Caldwell, E. F., and Grogg, B. Application of the thiobarbituric acid test to cereal and baked products. *Food Technol.,* **9**, 185 (1955).
7. Emmerie, A. and Engel, C. Colorimetric determination of dl-α-tocopherol (vitamin E). *Nature,* **142**, 873 (1938).
8. Fore, S. P., Morris, N. J., Mack, C. H., Freeman, A. F., and Bickford, W. G. Factors affecting the stability of crude oils of 16 varieties of peanuts. *J. Am. Oil Chemists' Soc.,* **30**, 298 (1953).
9. Colby, G. E. California walnuts, almonds, and chestnuts. *Biennial Rept. Calif. Agr. Expt. Sta.,* p. 142 (1896-1897).
10. Fang, S. C., and Bullis, D. E. Investigation of Barcelona and Du Chilly filbert nuts. 1. Chemical study of Barcelona and Du Chilly filbert nuts and oils. *J. Am. Oil Chemists' Soc.,* **26**, 512 (1949).
11. Griffiths, H. N., and Hilditch, T. P. Oleic-elaidic acid transformation as an aid in the analysis of mixtures of oleic, linoleic, and linolenic acids. *J. Soc. Chem. Ind.,* **53**, 75 (1934).
12. Henick, A. S., Benca, M. F., and Mitchell, J. H. Estimating carbonyl compounds in rancid fats and foods. *J. Am. Oil Chemists' Soc.,* **31**, 88 (1954).
13. Hove, E. L., and Harris, P. L. Note on the linoleic acid-tocopherol relationship in fats and oils. *J. Am. Chemists' Soc.,* **28**, 405 (1951).
14. Kenaston, C. B., Wilbur, K. M., Ottolenghi, A., and Bernheim, F. Comparison of methods for determining fatty acid oxidation produced by ultraviolet irradiation. *J. Am. Oil Chemists' Soc.,* **32**, 33 (1955).
15. Kester, E. B. Minor oil-producing crops of the United States. *J. Am. Oil Chemists' Soc.,* **26**, 65 (1949).
16. Lambert, M., and Neish, A. C. Rapid method for estimation of glycerol in fermentation solutions. *Can. J. Research,* **B28**, 83 (1950).
17. Lange, W. Cholesterol, phytosterol, and tocopherol content of food products and animal tissues. *J. Am. Oil Chemists' Soc.,* **27**, 414 (1950).
18. Luchinger, W. W., Cuendet, L. S., Boyer, P. D., and Geddes, W. F. A sensitive method for measuring lipase activity and its application to wheat products. *Cereal Chem.,* **32**, 395 (1955).
19. Musco, D. D., and Cruess, W. V. Food rancidity. Studies on deterioration of walnut meats. *J. Ag. Food Chem.,* **2**, 520 (1954).
20. Rockland, L. B. Recent progress on the utilization of shelled walnuts. *Diamond Walnut News,* **37**, (5) (1955).
21. Shifrin, KH. B. Biochemistry of walnuts [walnut oil]. *Biokhem. Kul'tur Rastenii.* **7**, 421 (1940); through *Chem. Abstr.,* **35**, 3114.
22. Siddiqi, A. M., and Tappel, A. L. Comparison of some lipoxidases and their mechanism of action. *J. Am. Oil Chemists' Soc.* (In press).
23. Sidwell, C. G., Salwain, H., and Mitchell, J. H. Measurement of oxidation in

dried milk products with thiobarbituric acid. *J. Am. Oil Chemists' Soc.*, **32**, 13 (1955).

24. TAPPEL, A. L. Linoleate oxidation catalysts occurring in animal tissues. ***Food Research***, **18**, 104 (1953).
25. TURNER, E. W., PAYNTER, W. D., MONTIE, E. J., BESSERT, M. W., STRUCK, G. M., AND OLSON, F. C. The use of the 2-thiobarbituric acid reagent to measure rancidity in frozen pork. *Food Technol.*, **8**, 326 (1954).
26. WIEGAND, E. H. Nut futures in the Pacific Northwest. *Ore. Sta. Hort. Soc. Ann. Rept.*, **35**, 107 (1943).
27. WILLARD, C., ENGLERT, R. D., AND RICHARDS, L. M. Fatty acid contents of several food products. *J. Am. Oil Chemists' Soc.*, **31**, 135 (1954).

BITTER FLAVOR IN CARROTS. III. THE ISOLATION OF A COMPOUND WITH SPECTRAL CHARACTERISTICS SIMILAR TO HYDROCARBON EXTRACTS OF BITTER CARROTS.[a]

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(Manuscript received December 26, 1956)

[a] Approved as Journal Paper No. 1055 New York State Agricultural Experiment Station, Geneva, N. Y.

In earlier publications *(1, 7)* it has been shown that the hydrocarbon extracts from bitter carrots contain ultraviolet absorbing material absent in extracts from non-bitter carrots. Good correlation was obtained between the spectrophotometric data and taste scores of 200 samples. By extraction with hexane or cyclohexane a crystalline substance, designated Compound A, has been isolated from bitter carrot roots. Compound A approximates the ultraviolet spectral characteristics of extracts of bitter carrots. This is shown in Figure 1. Dodson, Fukui, Ball, Carolus and Sell have recently reported the isolation of a compound from bitter carrots melting at 77° C. *(4)*. Probably this material is identical with Compound A. However, their suggested empirical formula of $C_{15}H_{15}O_5$ must be erroneous since no compound containing only carbon, hydrogen, and oxygen can have an odd number of hydrogen atoms.

Compound A is a colorless crystalline substance, melting at 76°. In the carrots we examined, its concentration varied from zero to 50 mg. per 100 g. fresh weight. Compound A has a low solubility in water and in 1 *N* hydrochloric acid but is readily soluble in ethanol, acetone, chloroform, carbon

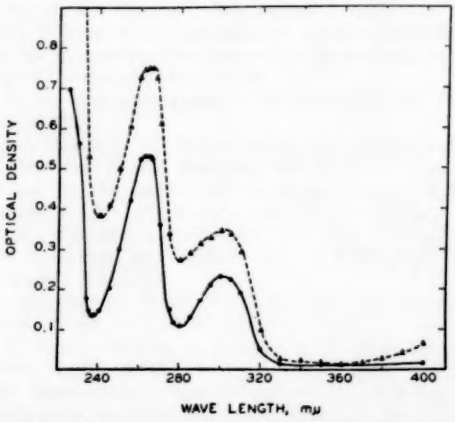

Figure 1. Ultraviolet absorption spectrum of the cyclohexane extract from bitter carrots. △--△, and of the crystalline compound, in cyclohexane, isolated from bitter carrots •———•, concentration 8.1 mg. per liter.

tetrachloride as well as in 1 *N* aqueous sodium hydroxide. The material is optically active, $[\alpha]_D^{24} - 56°$ (*c* 1 in methanol), and exhibits a strong greenish blue fluorescence when exposed to ultraviolet light.

Elemental analyses and molecular weight determination indicate an empirical formula of $C_{11}H_{12}O_4$ for Compound A. Other analyses indicate the presence of one alkoxy group, one "active hydrogen" and one or two C-methyl groups. The observation that compound A gives a violet color with ferric chloride, a positive Millon test, and an orange dye with diazotized sulfanilic acid is interpreted as strong evidence for the presence of a phenolic hydroxyl group. The azo dye is a suitable derivative of Compound A for chromatographic analysis.

In the previously published procedure *(7)* for the estimation of bitter flavor in carrots, the results were expressed in terms of "height of the 265 mμ peak." At that time the compound responsible for the absorption characteristics of the hydrocarbon extracts had not been isolated. The availability of pure Compound A makes it now possible to express these data as mg. Compound A per 100 g. sample by multiplying the value for the "height of the 265 mμ peak" by 2.2. Quantitative isolation of Compound A affords good evidence that Compound A is the major component of the hydrocarbon extract from bitter carrots having an absorption peak at 265 mμ. From two samples of bitter carrots in which the height of the 265 mμ peak was 3.5 (equivalent to 7.7 mg. Compound A per 100 g. sample), 7.8 and 5.2 mg. per 100 g. sample of Compound A could be isolated.

The fact that Compound A fluoresces is of interest since Bessey, Carolus and Sell *(2)* have shown that fluorescence measurements correlated well with bitter flavor in carrots. They observed a greater concentration of the fluorescing material in the phloem than in the xylem. Since we have also found larger amounts of the ultraviolet absorbing material, as measured by absorption spectra, in the phloem, it is suggested that the same compound is being determined by the two methods.

The relation of Compound A to the bitter flavor of carrots has been investigated. When bitter carrots are steam-distilled a bitter tasting distillate, containing oil droplets, is obtained. The concentration of Compound A in this distillate must be low since ultraviolet absorption measurements failed to show the presence of the material, Figure 2. However, the presence of Compound A could be easily demonstrated in the residue after steam distillation. The residue still possessed a bitter flavor. Steam distillation of the crystalline Compound A also showed that the material is only very slightly volatile in steam. That bitter carrots contain off-flavored steam volatile constituents has also been shown by Truscott *(8)*.

When the recrystallized Compound A is placed directly on the tongue, it does not exhibit a typical bitter taste. However, a 0.01% aqueous solution was found to be bitter by the taste panel. Further taste tests showed that the addition of Compound A to steam-distilled non-bitter carrots can be detected organoleptically. In addition, tasters could not distinguish between steam-distilled non-bitter carrots to which 0.02% Compound A had been added and steam-distilled bitter carrots.

The taste characteristics of Compound A are not unique since Higby *(6)* has shown that limonin, a bitter constituent of navel oranges, does not

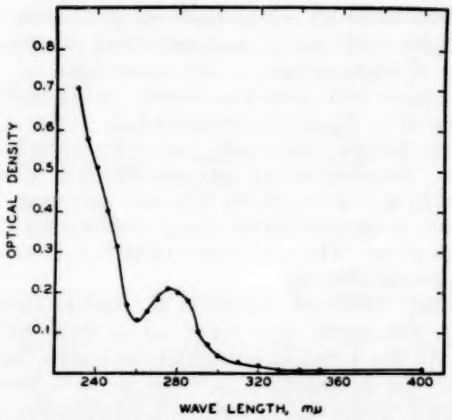

Figure 2. Ultraviolet absorption spectrum of steam distillate from bitter carrots. The distillate was diluted with ethanol.

taste bitter as a solid, but exhibits a pronounced bitter taste when it is dissolved in dilute alcohol.

It is therefore tentatively concluded that the bitter off-flavor of carrots is caused by the presence of several compounds and that Compound A is one of these. Although it is unlikely that the flavor characteristics attributed to Compound A are due to trace contaminants, this possibility cannot be excluded at the present time.

EXPERIMENTAL [b]

Isolation of Compound A. Carrots which were canned by the previously published method *(7)* and had been selected for bitter flavor were used for the isolation of Compound A. After macerating 10 kg. carrots in the blender they were extracted twice with 7 liters commercial n-hexane. The extracts were combined, filtered and concentrated at atmospheric pressure to 400 ml. The residue was transferred to a smaller flask and concentrated in vacuum to 25 ml. Crystals precipitated during the evaporation. After storage at 0° C. overnight and filtration 1.26 g. crude product was obtained. The orange-colored filtrate has the typical off-flavor of bitter carrots. After two recrystallizations from 40 ml. purified Skellysolve B *(5)*, the yield of Compound A melting at 76° was 0.980 g. Material recrystallized several times from aqueous methanol had the same melting point. In cyclohexane the compound has two absorption maxima, one at 300 mμ $E^{1\%}_{1\,cm.} = 300$ and another one at 264 mμ $E^{1\%}_{1\,cm.} = 655$.

Anal. Calcd. for $C_{11}H_{12}O_4$; C, 63.4; H, 5.81; O, 30.8; mol. wt. 208; OCH_3, 14.9; active H, 0.48; $C-CH_3$, 7.2 for 1 or 14.4 for 2 $C-CH_3$ groups. *Found:* C, 63.4; H, 5.78; O, 32.9; mol. wt. (Rast) 183; OCH_3, 16.9; active H, 0.48; $C-CH_3$, 9.3 (Kuhn, Roth).

Coupling of Compound A with diazotized sulfanilic acid. This deriva-

[b] All melting points were determined on a microscope hot-stage and are corrected. Analyses are by Dr. G. Weiler and Dr. F. B. Strauss, Oxford, England.

tive was prepared by the procedure of Chang, Hossfelt and Sandstrom *(3)*. From 54 mg. Compound A, 75 mg. orange needles were obtained which did not melt below 250°. The sodium salt of the dye in methanol has an absorption peak at 395 mμ. The pigment was chromatogrammed on Whatman paper No. 1 which had been dipped in 2% sodium carbonate solution *(2)*, and gave an R_f 0.27 with n-butanol, ethanol, water (5 : 1.75 : 5) and an R_f of 0.13 with secondary butanol saturated with 2% aqueous sodium carbonate.

SUMMARY

A crystalline substance has been isolated from bitter carrots which approximates the ultraviolet spectral characteristics of hydrocarbon extracts from the bitter carrots. Some of the physical properties and analytical data of the compound are reported. An empirical formula of $C_{11}H_{12}O_4$ is suggested for the compound. Evidence is presented that the bitter off-flavor of carrots is due to several components and that the isolated crystalline compound is one of these.

Addendum

(Added to proof, June 10, 1957)

Compound A has been identified by us as 3-methyl-6-methoxy-8-hydroxy-3,4-dihydroisocoumarin.—E. S.

LITERATURE CITED

1. Atkins, J. D. Bitter flavor in carrots. II. Progress report on field and storage experiments. Bulletin No. 774, 1956. New York State Agricultural Experiment Station, Geneva, N. Y.
2. Bessey, P. M., Carolus, R. L., and Sell, M. H. Fluorescence and ethylene as related to bitterness in carrots. Abstract of Papers, American Society for Horticultural Science, Aug. 27-29, 1956, page 2a.
3. Chang, W. H., Hossfeld, H. L., and Sandstrom, Wm. M. Identification of phenols by paper partition chromatography. *J. Am. Chem. Soc.*, **74**, 5766 (1952).
4. Dodson, A., Fukui, H. N., Ball, C. D., Carolus, R. L., and Sell, H. M. Occurrence of a bitter principle in carrots. *Science*, **124**, 984 (1956).
5. Graff, H. H., O'Connor, K. T., and Skan, E. L. Purification of solvents for absorption spectroscopy. *Anal. Chem.*, **16**, 556 (1944).
6. Higby, R. H. Bitter constituents of navel and Valencia oranges. *J. Am. Chem. Soc.*, **60**, 3013 (1938).
7. Sondheimer, E., Phillips, W. F., and Atkin, J. D. Bitter flavor in carrots. I. A tentative spectrophotometric method for the estimation of bitterness. *Food Research*, **20**, 659 (1955).
8. Truscott, J. H. L. Bitter flavour in stored carrots. Report of the Horticultural Products Laboratory. (Vineland, Ontario, Canada), 108 (1953-1954).

TEXTURE CHANGES IN FROZEN MONTMORENCY CHERRIES

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In connection with the time-temperature tolerance studies on frozen fruits conducted at this Laboratory, it was noted that frozen Montmorency cherries toughened when they were stored at temperatures of 20° F. or higher but were stable at —10° F. Toughening of texture was noticeable after 2 weeks' storage and progressively increased with longer periods of time.

It was thought that the change in texture might be related to a change in the chemical characteristics of the pectic substances. A procedure for characterizing pectic substances without extraction from the fruit tissue was used.[a] Quantitative methods of analyses were applied to cherry marc (insoluble residue) prepared by extraction of the fruit with alcohol.

MATERIALS AND METHODS

Commercially packed Montmorency cherries from Traverse City, Michigan, and experimental samples from the U. S. Fruit and Vegetable Products Laboratory of this Division at Puyallup, Washington, were used in the following studies. The experimental cherries were processed in the following way. Freshly harvested Montmorency cherries were soaked in water at 45° F. for 12 hours. The cherries were sorted to remove damaged fruit, pitted and frozen to −10° F. with 60° B. sirup and without sirup in air. A description of samples used is presented in Table 1.

TABLE 1

Analyses of Montmorency cherries

Sample	Storage	Source	Anhydrouronic acid in marc	Esterification of pectic substances	Tenderometer readings
			%	%	
Untreated	−10° F.	Wash., 1955	32	31	37
Siruped	−10° F., control	Wash., 1955	33	30	36
Siruped	6 weeks at 20° F.	Wash., 1955	34	21	58
Untreated	−10° F., control	Wash., 1956	33	27	42
Untreated	5 days at 34° F.	Wash., 1956	29	18	75
Siruped	−10° F., control	Mich., 1953 [1]	30	32	46
Siruped	4 weeks at 20° F.	Mich., 1953 [1]	33	21	68
Siruped	−10° F., control	Mich., 1953	32	32	54
Siruped	4 weeks at 20° F.	Mich., 1953	31	26	63

[1] Immature fruit.

Cherries stored at −10° F. were frozen solid but at 20° F. the fruit and its sirup had the consistency of an icy slush.

After storage the cherries were thawed to 65° F. and drained. Tenderometer readings, pectinesterase, and solids and calcium analyses were made directly on the drained

[a] Details of the procedure will be published in the near future.

fruit. Tenderometer values were read on scale 2.[b] Pectinesterase activity was determined by the method of MacDonnell, Jansen, and Lineweaver *(2)*.

Cherry marc was prepared by blending the drained cherries with 3 volumes of 95% ethanol in an electric blender and allowing the slurry to stand at room temperature for one hour. The pulp was suction filtered on Eaton-Dikeman No. 616 paper and the filter cake was then suspended in 2 volumes of a 5% solution of hydrochloric acid in 80% ethanol for one hour at room temperature. The acid treatment was used to remove salts in order to free carboxyl groups for titration with alkali. After filtration the marc was washed with 70% ethanol until a negative chloride test with silver nitrate solution was obtained. The pressed cake was washed twice with acetone and dried overnight in air without heating. The dried marc was ground in a Wiley mill to pass a 40-mesh screen.

Moisture, ash, acetyl, anhydrouronic acid, and per cent esterification analyses were made on the cherry marc. Moistures were run at 60° C. for 20 hours *in vacuo*. Samples were ashed at 600° C. for 4 hours to constant weight. Acetyl determinations were made by a colorimetric procedure *(3)* and the pectic substances characterized by titration of the marc.

A 1-g. sample of dried marc was suspended in 100 ml. of distilled water containing 1 g. of sodium chloride. Free carboxyls were determined by titration of the slurry with 0.1 *N* sodium hydroxide until the pink color of Hinton's indicator *(1)* end-point held for at least one minute. Samples were shaken vigorously during the titration. This titer was expressed in milliequivalents of free carboxyls. The saponification equivalent was determined on the samples after titration of the free carboxyls. A pipetted sample of 10 ml. of 0.500 *N* sodium hydroxide was added to the slurry and the mixture allowed to stand at room temperature for 30 minutes to saponify the *-O*-acetyl and carbomethoxyl groups. Ten milliliters of 0.500 *N* hydrochloric acid was pipetted into the titration flask and the liberated carboxyls were titrated with 0.1 *N* sodium hydroxide to the pink end-point of Hinton's indicator *(1)*. The titer of the liberated carboxyls gave a value for the sum of the carbomethoxyls and the *-O*-acetyls present in the marc. Calculations were made with the following formulas:

$$\text{Meq. carbomethoxyl per gram} = \text{saponification meq. per gram} - \text{meq. } \textit{-O}\text{-acetyl per gram.}$$

$$\text{Meq. uronic acid per gram} = \text{meq. free carboxyls per gram} + \text{meq. carbomethoxyls per gram.}$$

$$\text{Percent esterification} = \frac{\text{meq. carbomethoxyls per gram} \times 100}{\text{meq. uronic acid per gram}}.$$

$$\text{Percent anhydrouronic acid} = \frac{\text{meq. uronic acid per gram} \times 176 \times 100}{1{,}000}.$$

RESULTS AND DISCUSSION

All cherry samples stored at 20° F. or higher exhibited a texture change. The toughening produced after 4 to 6 weeks at 20° F. did not alter the fruit sufficiently to make it unpalatable. There was no apparent shrinkage of fruit tissue after storage at elevated temperatures.

The drained cherry samples all had about 0.018% calcium on the fresh-weight basis. Total solids of the sugared samples was 18% and that of the untreated was 14%. Moistures of the cherry marc were about 11% and the ash varied from 0.6% to 1%. The alkalinity of the ash was negligible.

Toughening does not appear to be related to sugar treatment of the cherries. Siruped and untreated control samples gave the same tenderometer value after storage at −10° F. Siruped cherries that had been stored at

[b] The tenderometer instrument used for these measurements was built by the Food Machinery Corporation. Mention of products does not imply endorsement by the Department of Agriculture.

20° F. for 6 weeks were soaked in distilled water for 4 hours to reduce the sugar content by diffusion. Tenderometer readings were the same before and after soaking.

Analyses of the cherry marc are presented on a moisture- and ash-free basis in Table 1. The -*O*-acetyl values were all about 1% of the marc and were the same for the control and the cherries after storage. Pectinesterase activity of all samples was about 0.004 meq. of carbomethoxyl groups of pectin hydrolyzed per minute per gram fruit. Though the percentage of esterification of the control samples varied, all samples exhibited a drop in carbomethoxyls after storage at 20° F. or higher. This drop in esterification was accompanied by an increase in toughness as measured by tenderometer readings. As the temperature of storage was increased, the rate of toughening increased.

SUMMARY

Frozen Montmorency cherries harvested in Washington and Michigan were stored at −10° F. for long periods of time and at 20° F. or higher for periods of 5 days to 6 weeks. The cherries toughened during storage at elevated temperatures but the texture was stable when stored at −10° F. Characterization of the pectic substances showed a lower percentage of esterification of the toughened cherries. Change in texture may be attributed to an enzymic deesterification and the formation of a calcium pectinate gel from calcium present in the fruit tissue combining with carboxyl groups when the cherries are not frozen solid as they are at −10° F.

LITERATURE CITED

1. Hinton, C. L. *Fruit Pectins*, p. 27, 1940, Chemical Publishing Co., New York.
2. MacDonnell, L. R., Jansen, E. F., and Lineweaver, Hans. The properties of orange pectinesterase. *Arch. Biochem.*, 6, 389-401 (1945).
3. McComb, Elizabeth A., and McCready, R. M. Determination of acetyl in pectin and in acetylated carbohydrate polymers by the hydroxamic acid reaction. *Anal. Chem.*, 29, 819-821 (1957).

NUTRITIVE VALUE OF CENTRAL AMERICAN CORNS V. CAROTENE CONTENT AND VITAMIN A ACTIVITY OF THREE GUATEMALAN YELLOW CORNS

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In Central America corn is used extensively in animal feeds and may make up as high as 75% of human diets in rural areas. Avitaminosis A is a major nutritional problem of both humans and animals and estimation of vitamin A activity of feedstuffs is important. Bressani *et al. (4)* have shown that for this estimation, chemical methods must consider both cryptoxanthin and beta-carotene since local yellow corns contain significant amounts of both pigments.

In the present report further data are presented on the beta-carotene and cryptoxanthin content of three Guatemalan yellow corns and their vitamin A activity as shown by chemical determinations, as well as the growth, mortality, and blood serum levels of vitamin A and carotene of baby chicks.

MATERIALS AND METHODS

The characteristics of the three Guatemalan yellow corns, Tiquisate Golden Yellow (TGY), 142-48 and Santa María Cauqué (SMC), have been previously described *(3)*. Total carotenoid pigments were extracted with petroleum ether and then washed with 90% aqueous methanol to remove lutein and zeoxanthin. The carotenoid extract was first read at 440 mμ[c] and then passed through a magnesium oxide-celite column. The beta-carotene was eluted with 5% acetone in petroleum ether according to the method of Guilbert *(5)* as modified by Peterson *et al. (6)*, and the cryptoxanthin remaining in the column was eluted with 15% acetone in petroleum ether. Both fractions were read at 440 mμ. An absorption curve of the latter fraction was identical with that of cryptoxanthin. Total vitamin A activity was calculated on the basis of cryptoxanthin having one-fourth, and beta-carotene one-half, the activity of crystalline vitamin A.

For the biological trials, 3-day-old New Hampshire baby chicks were distributed by weight among 16 experimental groups. Each group contained 12 chicks and was housed in an all-wire cage with the temperature decreased progressively from 35° C. at 3 days to 21° C. at 5 weeks. The birds were weighed weekly during a 35-day period; the diets and water were fed *ad libitum* and feed consumption recorded daily.

The composition of the low vitamin A basal ration in grams per 100 grams was: sesame oil meal, 30; corozo oil meal,[d] 15; vitamin test casein, 4; ground sorghum, 47;

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[a] (IAN), a technical agricultural organization operated jointly by the Government of Guatemala and the International Cooperation Administration of the United States Government, through the Servicio Cooperativo Interamericano de Agricultura.

[b] (INCAP), a human nutrition Institute supported by the Governments of Central America and Panama and administered by the Pan American Sanitary Bureau, Regional Office of the World Health Organization. INCAP Scientific Publication No. I-68.

[c] Evelyn colorimeter.

[d] This is prepared from the nut of the palm *Orbignya cohume*.

mineral mixture with minor elements,[e] 3; APF,[f] 0.7; Delsterol, 0.02; and 0.5 ml. of an aqueous vitamin B-complex solution. Each corn was fed at 1, 3, 6, and 12% of the basal ration replacing part of the ground sorghum. The control rations contained 0, 30, 60, and 120 I. U. of vitamin A, added to each 100 grams of the basal ration.

At the end of the 5-week feeding period, each bird was bled by heart puncture and the blood samples refrigerated and centrifuged one hour later. The individual serum samples were analyzed for total carotenoids and vitamin A by the method of Bessey *et al. (2)*.

RESULTS

Total carotenoid, beta-carotene and cryptoxanthin of the 3 corn samples are given in Table 1, together with the calculated vitamin A activity. Total carotenoids were

TABLE 1

Total carotene, beta-carotene and cryptoxanthin content of three Guatemalan corns

Corn	Total carotene	Beta-carotene		Cryptoxanthin		Estimated vitamin A activity [1]
	mcg./g.	mcg./g.	% of total	mcg./g.	% of total	mcg./g.
TGY	11.28	3.00	26.5	4.33	38.3	2.58
142-48	8.87	1.95	21.9	5.04	56.8	2.24
SMC	6.37	1.22	19.2	3.65	57.3	1.52

[1] ½ beta-carotene + ¼ cryptoxanthin.

highest in the variety TGY (11.28 mcg./g.) and significantly lower in 142-48 (8.87 mcg./g.) and SMC (6.37 mcg./g.). The beta-carotene of TGY was 35% higher than that of 142-48 and 59% higher than that of SMC. However, variety 142-48 had the largest amount of cryptoxanthin (5.04 mcg./g.) with TGY (4.33 mcg./g.) next and SMC (3.65 mcg./g.) the lowest.

Weight in grams, efficiency of feed utilization and mortality of the chicks fed the various levels of the 3 corns are shown in Table 2. One hundred per cent mortality from avitaminosis A occurred in the chicks fed the unsupplemented basal ration. Sixty or more I. U. of vitamin A or 3% or more of each of the corns tested proved sufficient to prevent death from avitaminosis A under the conditions of the trial.

Rate of growth and efficiency of feed utilization were highest in the groups fed 142-48, with those fed SMC intermediate, and TGY the lowest. The growth rates of the chicks fed the control ration containing 30, 60, and 120 I. U. of vitamin A were similar to those of chicks fed the 6 and 12% levels of the corn varieties 142-48 and SMC and at all levels superior to the growth of the chicks fed TGY.

Data in Table 3 indicate that the blood serum carotenoids and vitamin A levels were significantly higher in the groups fed TGY than 142-48 and SMC. The blood serum, vitamin A, and carotene levels of the chicks fed the different corns showed a highly significant linear relationship to the various levels of carotene ($Y = 0.05X + 24.7$) and vitamin A ($Y = 0.004X + 0.20$) fed (Figure 1). The 900 mcg. standard fell exactly on the serum vitamin A regression line and that for 1800 and 3600 mcg. below it. The carotene vitamin A ratio was similar in the groups fed 142-48 and SMC and approximately twice as high in the groups fed TGY.

DISCUSSION

By calculation the chemical data indicate that all three of the varieties of yellow corn should contain sufficient vitamin A activity for the physical

[e] This mixture consisted of one part calcium carbonate, one part sodium chloride, one part bonemeal and one-tenth part of the minor element mixture "Mini-Rich" produced by the Thompson-Hayward Chemical Company.

[f] Aureofac, courtesy of Dr. T. H. Jukes and the Lederle Laboratories, N. Y.

TABLE 2
Vitamin A activity, growth, mortality, feed efficiency data and vitamin A and total carotene levels in blood serum

Ration	No. chicks at end [1]	Final weight grams	Efficiency feed utilization
Control (Amount of vitamin A)			
0 mcg. (0 units)	0	—	—
900 mcg. (30 units)	11	323	2.66
1800 mcg. (60 units)	12	377	2.20
3600 mcg. (120 units)	12	367	2.26
Corn TGY (Proportion fed)			
1%	10	229	3.50
3%	12	257	2.98
6%	12	304	2.84
12%	12	295	2.86
Corn 142-48			
1%	9	269	3.30
3%	12	294	2.44
6%	12	318	2.32
12%	11	381	2.30
Corn SMC			
1%	8	257	4.22
3%	12	281	2.62
6%	12	324	2.54
12%	12	347	2.45

[1] All groups began with 12 chicks.

well-being of baby chicks. However, the vitamin A activity is contributed largely by cryptoxanthin and beta-carotene and the relative amounts of each are not constant among different corns. This further emphasizes the importance of analyzing corn for both cryptoxanthin and beta-carotene.

The biological assays with chicks showed that when 3% of any of the three corns studied was fed in the basal ration, no mortality occurred. In the case of both 142-48 and SMC the higher the level fed within the range studied, the greater the weight gain and the better the feed efficiency. However, with corn variety TGY the growth and feed efficiency at the highest level fed was actually poorer than at an intermediate level. Although no

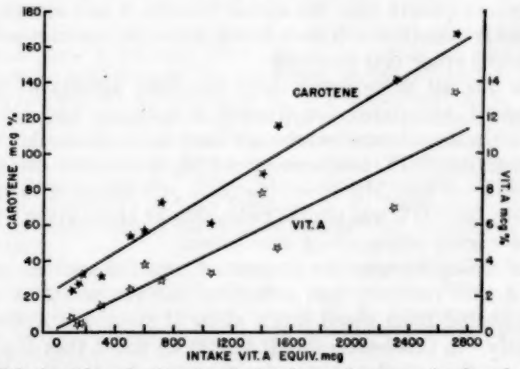

Figure 1. Relationship of vitamin A intake and serum levels of carotene and vitamin A.

TABLE 3
Effect of 3 Guatemalan corns on the blood serum vitamin A and carotene levels of New Hampshire baby chicks

Ration	Dietary Intake				Blood Serum	
	Total carotene	Cryptoxanthin	Beta-carotene	Vitamin A equivalent[1]	Carotene	Vitamin A
%	*mg.*	*mg.*	*mg.*	*mg.*	*mcg./100 ml.*	*mcg./100 ml.*
TGY						
1	0.72	0.27	0.19	0.16	33	0.46
3	2.67	1.01	0.70	0.60	57	3.78
6	6.10	2.34	1.62	1.40	88	7.75
12	11.88	4.56	3.15	2.72	167	13.42
142						
1	0.57	0.32	0.13	0.14	27	0.40
3	1.99	1.13	0.44	0.50	54	2.40
6	4.13	2.35	0.91	1.04	61	3.30
12	9.09	5.17	2.00	2.29	140	6.90
SMC						
1	0.43	0.25	0.08	0.10	23	0.85
3	1.31	0.75	0.25	0.31	31	1.50
6	3.01	1.73	0.58	0.72	73	2.90
12	6.27	3.59	1.20	1.50	116	4.70
Vitamin A (Standard)						
0 I.U.	.....	.....	.....	0.00	....	.....
30 I.U.	.....	.....	.....	0.90	28	3.80
60 I.U.	.....	.....	.....	1.80	25	5.50
120 I.U.	.....	.....	.....	3.60	23	10.20

[1] ½ beta carotene + ¼ cryptoxanthin

certain explanation can be given for this depression of growth in chicks fed the highest level of TGY in this particular trial, it is possible that it was due to treatment with Arasan[5] which this sample alone received. Ackerson and Mussehl *(1)* have reported growth depression in chicks fed corn treated with either Arasan or Orthocide and Waibel, Pomeroy and Johnson *(9)* have shown that Arasan-treated corn had a very pronounced depressing effect on egg production. The data for TGY are included because, despite the comparatively poorer growth rate, the serum vitamin A and carotene levels show the same highly significant linear trend with the amount of corn fed as observed for the other two varieties.

The data for all three corns show excellent agreement between their carotene content, calculated for vitamin A activity, and the blood serum levels of vitamin A and total carotene. The corn with the highest estimated vitamin A activity, TGY, maintained the highest serum carotene and vitamin A at each level fed. Moreover, the ratio of vitamin A to carotene in the blood of chicks fed TGY was almost twice that of those given 142-48 or SMC, suggesting a higher efficiency of conversion.

The relationship between the amount of corn fed and the resulting levels of vitamin A and carotene was such that the vitamin A activity of corn could be estimated from blood levels alone if suitable standards were run simultaneously. In this connection it should be noted that the first standard fell exactly on the calculated line for the vitamin A activity of the cereal meals determined by chemical analysis and that the intercept of this line

was essentially zero. The two higher standards fell somewhat below the calculated line suggesting that at higher dosage levels the vitamin A standard was less efficiently absorbed than the carotene in the corns.

The intercept of 24.7 mcg. for the serum carotene regression line was close to the lower limit of serum carotene observed in previous depletion experiments *(7, 8)*. It is useful to call attention again to the inadequacy of estimating vitamin A activity of many plants from the determination of a single carotene such as beta-carotene or cryptoxanthin. Accurate chemical estimations of vitamin A activity require the analysis of all of the active carotenes which may be present along with the assigning of a proper factor for their relative activity.

SUMMARY

Three varieties of yellow corn grown in Guatemala were found by chemical analysis to have the following vitamin A activity: TGY 2.58 mcg./g.; 142-48, 2.24 mcg./g.; and SMC, 1.52 mcg./g. Of this activity, 38.3, 56.8 and 57.3% respectively, was accounted for by cryptoxanthin and the remainder by beta-carotene. Other carotene pigments made up 35.2, 21.3 and 23.5%, respectively, of the total carotene measured. Three per cent of these varieties added to the basal vitamin A deficient ration of baby chicks sufficed to prevent mortality and improve growth. Within the range of 0 to 12% studied blood serum carotene and vitamin A values increased linearly with the amount of corn fed. Highest absolute blood levels and the highest ratio of carotene to vitamin A in the serum were observed for corn variety TGY. The importance of analyzing corn for both beta-carotene and cryptoxanthin is emphasized.

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[4] Fungicide for disinfection of seeds produced by Du Pont de Nemours and Company.

LITERATURE CITED

1. Ackerson, C. W., and Mussehl, F. E. Toxicity of treated corn in rations for chicks. *Poultry Sci.*, **34**, 728-729 (1955).
2. Bessey, O. A., Lowry, O. H., Brock, M. J., and Lopez, J. A. The determination of vitamin A and carotene in small quantities of blood serum. *J. Biol. Chem.*, **166**, 177-188 (1946).
3. Bressani, R., Arroyave, G., and Scrimshaw, N. S. The nutritive value of Central American Corns. I. Nitrogen, ether extract, crude fiber and minerals of twenty-four varieties in Guatemala. *Food Research*, **18**, 261-267 (1953).
4. Bressani, R., Campos, A. A., Squibb, R. L., and Scrimshaw, N. S. The nutritive value of Central American corns. IV. The carotene content of thirty-two selections of Guatemalan corn. *Food Research*, **18**, 618-624 (1953).
5. Guilbert, H. R. Determination of carotene as a means of estimating the vitamin A value of forage. *Ind. Eng. Chem., Anal. Ed.*, **6**, 452-454 (1934).
6. Peterson, W. J., Hughes, J. S., and Freeman, H. F. Determination of carotene in forage. A modification of the Guilbert method. *Ind. Eng. Chem., Anal. Ed.*, **9**, 71-72 (1937).
7. Squibb, R. L., and Braham, J. E. Blood meal as a lysine supplement to all-vegetable protein rations for chicks. *Poultry Sci.*, **34**, 1050-1053 (1955).
8. Squibb, R. L., Braham, J. E., and Scrimshaw, N. S. Absorption of vitamin A in aqueous and oil solutions by depleted New Hampshire chicks. *Poultry Sci.*, **35**, 73-76 (1956).
9. Waibel, P. E., Pomeroy, B. S., and Johnson, E. L. Effect of Arasan-treated corn on laying hens. *Science*, **121**, 401-402 (1955).

EFFECT OF COPPER CHELATING AGENTS ON THE PRO-OXIDANT ACTIVITY OF ASCORBIC ACID WITH UNSATURATED FATS[a,b]

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Ascorbic acid, a strong reducing agent, catalyzes the formation of nitric oxide myoglobin with or without the use of heat *(25, 22)* and is effective in protecting the cured meat pigment once it is formed. In view of the usefulness of this compound in preserving meat color, its behavior with tissue fats becomes of particular interest. Ascorbic acid has been reported to act as an antioxidant in pure fats *(6, 13)*. However, it may also accelerate oxidation especially when added to animal fats in contact with an aqueous phase *(1, 21)*. Ascorbyl palmitate, which is fat soluble, oxidizes fat alone *(17)*. In numerous experiments in this laboratory in which ascorbic acid has been added to meats, it has accelerated rancidity in the majority of cases, but occasionally, in some samples of meat, has acted as an antioxidant.

The mechanism of accelerated fat oxidation by this compound is not clear. Indirect evidence points to a copper-ascorbic acid combination as the causative agent for the pro-oxidation. Lehmann and Watts *(15)* found that when ethylenediaminetetraacetic acid or polyphosphates, which form copper chelates, were added with ascorbic acid, its pro-oxidant effect in aqueous fat systems was eliminated. It was further found that certain phenolic antioxidants, such as with nordihydroguaiaretic acid *(26)* and liquid smokes *(24)* were synergistic with ascorbic acid which resulted in powerful antioxidant combinations. However, other polyhydroxy primary antioxidants did not act as antioxidants in aqueous fat systems to which ascorbic acid had been added.

A number of investigators have shown that substanees naturally occurring in plants can inhibit the oxidation of fats *(3, 7, 14)*. This may be due to the flavonoid components of these plants since it has veen shown *(8, 20)* that these substances can chelate with copper and diminish its catalytic effect. It has been shown *(8, 16)* that structural modifications were responsible for large differences in chelating ability.

It was of interest, therefore, to ascertain the effectiveness of these and other polyhydric compounds as antioxidants in aqueous fat systems to which ascorbic acid had been added and to compare their antioxidant effect with their copper chelating activity.

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[a] Financed in part by a grant from Swift and Co. to the Florida State University.

[b] Based on the dissertation by G. G. Kelley, offered in partial fulfillment of the Ph.D. degree at Florida State University.

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METHODS

Antioxidant evaluation. In order to obtain a rapid evaluation of the relative antioxidant effect of the various compounds, an accelerated test was performed using artificial aqueous fat systems. The method has been described in detail elsewhere *(21, 15)*. One milliliter of the aqueous solution containing the compound to be tested in 0.02 M phosphate buffer, pH 5.8, was absorbed onto a slightly smaller filter paper which was carefully placed over the first, eliminating all air bubbles. The petri dishes were sealed and stored in an incubator at 45° C. The melted fat very quickly distributes evenly over both papers in intimate contact with the aqueous solution. Peroxidation is accompanied by bleaching of the carotene, and the samples were designated rancid when the carotene was approximately half bleached as determined by visual inspection. Each of the compounds was tested alone and in combination with ascorbic acid.

Previous work *(4, 25)* has shown that concentrations of ascorbic acid within the range 0.02 to 0.1% were sufficient to catalyze the production of the cured meat pigment, and to stabilize the color of fresh meat under certain conditions. A concentration of 0.03% ascorbic acid was used throughout these experiments. The water soluble test compounds were added to the aqueous phase in a final concentration of 0.01%. All components were adjusted to pH 5.8.

Those compounds which had low solubility in water such as nordihydroguaiaretic acid and quercetin were dissolved in boiling water and while still hot they were diluted to their final concentration. No recrystallization was noted, and the tests were carried out immediately after the final dilution was completed.

Copper chelation. When a copper salt reacts with ascorbic acid it must be in the cupric form. The cupric chelate of ascorbate, by an electron shift to the metal, acquires a semiquinone structure. There then follows a dissociation since monovalent copper does not have a strong affinity for oxygen donors. *(16, 18, 27)*. Cuproin (2,2′-biquinolyl) combines with the cuprous ion to form a colored complex which can be detected and whose concentration can be measured by the absorption at 545 mμ with the Beckman DU spectrophotometer. Cuproin prepared in a 50% (volume) aqueous ethanol solvent system to a final concentration of 1% provided cuproin solubility and a highly aqueous polar medium *(11)*. Ascorbic acid, in the presence of cupric ions and cuproin, catalyzes the production of the colored cuprous-cuproin complex, giving maximum optical density in less than 30 seconds. The effectiveness of added compounds in inhibiting the formation of this complex was measured by plotting the increase in optical density as a function of time. The time to reach an optical density of 50% of the maximum with ascorbic acid and cuproin alone was assumed to be the time required to reduce 50% of the cupric ion to the cuprous state. Any increase in the time required to reach this point could be taken as a measure of the ability of the compound under study to form a stable chelate with copper.

The solutions used in the spectrophotometric method for following the production of cuprous ion are described in detail below. The water used to make up the various concentrations was triple distilled in glass and demineralized. All concentrations are given as final concentrations.

Four solutions were prepared for each test. Each contained equal parts of ascorbic acid (1×10^{-4}M), 0.1% cuproin, 50% ethanol, to which can be added 1×10^{-5}M of test compound, and 0.01M trishydroxyaminomethane buffer, which serves the dual purpose of buffering the solution at pH 5.8 and acting as a vehicle for the addition of cupric chloride (1×10^{-6}M cupric ion). Solution number one, from which the copper was withheld, was used to indicate the presence of contaminating cupric ions. Solution number two, containing all addenda except the test substance, was used to obtain the maximum optical density. Three and four (duplicates) containing all constituents, were used to determine the relative values of copper chelation by test substances. In all cases the reaction was initiated by addition of the ascorbic acid to the other addenda and the timing recorded from that point.

Detection of trace amounts of copper in test solutions. According to Feigl *(10)*, the red-violet aqueous alkaline solution of 1,2-diaminoanthroquinone-3 sulfonic acid turns deep blue on addition of small amounts of copper salts. A drop of the neutral or acid test solutions on a spot plate, or in a microcrucible, is treated with a drop of the reagent

and then made alkaline with 1N sodium hydroxide. If the color changes from red violet to deep blue, copper is present. Copper can be positively identified in concentrations as low as 0.02 gamma per ml.

RESULTS

The triple distilled water, buffer solution, and fat gave positive tests for copper. Although copper was present in only trace amounts in each of the addenda tested, the total copper present in the aqueous fat system could be appreciable.

The data on the relative effectiveness of various types of compounds in promoting or preventing rancidity when alone and in combination with ascorbic acid are given in Table 1. It can be seen that all compounds tested, with the exception of naringenin and hesperidin, neither of which have ortho- or para-hydroxy groups, had some antioxidant effect when used alone. However, of those compounds tested which had antioxidant activity alone, only those with marked ability to chelate copper synergized with ascorbic acid.

TABLE 1

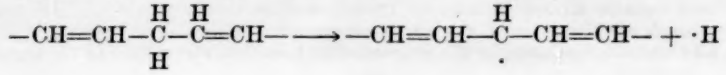
Antioxidant and prooxidant effect of some polyhydroxy compounds

Compound Added	Days to turn rancid at 45°C.		Ability to chelate copper [1]
	No ascorbic acid	Ascorbic acid 0.03%	
Control buffer pH 5.8	8	< 0.5	< 30 secs.
Nordihydroguaiaretic acid	30	44	3 hrs.
Quercetin	33	64	4 hrs.
Myricitin	31	61	4 hrs.
Quercetrin	28	38	3 hrs.
Luteolin	23	31	3 hrs.
Rutin	22	28	3 hrs.
Gallocatechin	21	28	3 hrs.
Cyanidin	21	26	3 hrs.
Catechol	21	< 0.5	< 30 secs.
Hydroquinone	21	< 0.5	< 30 secs.
Propyl gallate	21	< 0.5	< 30 secs.
Esculin	12	< 0.5	< 30 secs.
Naringenin	8	< 0.5	< 30 secs.
Hesperidin	8	< 0.5	< 30 secs.

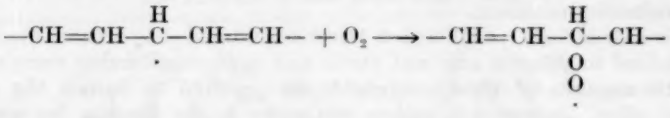
[1] Delay (presumed to be due to copper complexing) in time to reach 50% reduction of cupric ion by ascorbic acid.

DISCUSSION

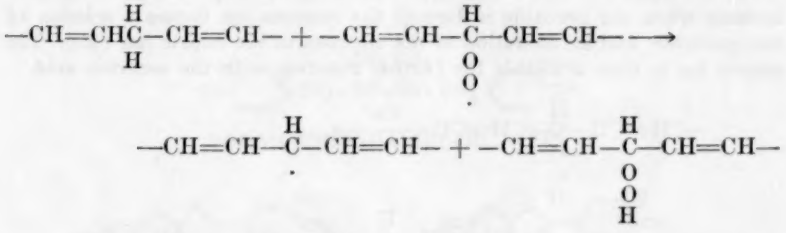
An hypothesis concerning the mechanism of the pro-oxidant effect of ascorbic acid. Rancidity results from the oxidative decomposition of unsaturated fats. Non-conjugated polyethenoic acids are especially susceptible to oxidative rancidity due to the activation of methylene groups by two adjacent double bonds. It has been found that although ascorbic acid incorporated into an aqueous fat phase exerts a powerful pro-oxidant effect, this effect is confined to fats containing such an active methylene group *(26)*. The first step in the oxidative decomposition of unsaturated fats is believed to be a dehydrogenation from this activated methylene group to yield a free radical.



There then follows the addition of oxygen to the methylene radical.



This radical in turn brings about a dehydrogenation from an activated methylene group of another fat molecule, and the cycle is continuous.



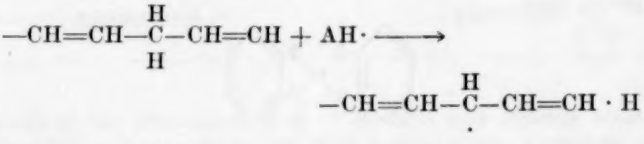
Decomposition of peroxides may produce additional free radicals initiating more chains.

The time required for oxidation of fat in an aqueous fat system is considerably shorter than that required for the same system to which ascorbic acid has been added. It may be assumed that the ascorbic acid is involved in initiation of the primary oxidative step in fat rancidity, i.e., the dehydrogenation of the activated methylene group.

It has been shown *(18, 27)* that copper catalyzes the oxidation of ascorbic acid (AH_2) with the formation of a free radical as follows:

$$AH_2 \longrightarrow AH^- + H^+$$
$$AH^- + Cu^{++} \longrightarrow AH\cdot + Cu^+$$

If the further assumption is made that the $AH\cdot$ can cause the dehydrogenation of the activated methylene group of the fat, this could be the initiation step of the peroxidation cycle.



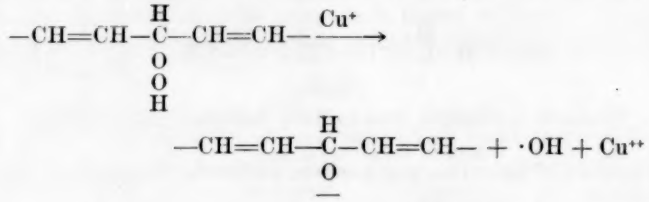
In the absence of experimental evidence on the disappearance of ascorbic acid from an aqueous fat solution, it may be postulated that ascorbic acid can either (a) recombine: $AH\cdot + \cdot H \longrightarrow AH_2$ or (b) disproportionate: $2AH\cdot \longrightarrow AH_2 + A$, or both.

It had been found in earlier work *(23)* that dehydroascorbic acid was also capable of initiating the very rapid oxidation of fat in an aqueous system. Since then it has been shown *(9, 19)* that under certain condi-

tions ascorbic acid can be regenerated from dehydroascorbic acid by oxido-reduction reactions.

It is also entirely possible that ascorbic acid and dehydroascorbic acid are oxidized to threonic acid and oxalic acid since considerably more than catalytic amounts of these compounds are required to initiate the prooxidant effect. In contrast, copper was active in the aqueous fat system in catalytic amounts of 1×10^{-6} M. Copper concentrations of this magnitude were normally found in this system.

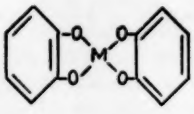
It is reasonable that copper is necessary in only catalytic amounts because when the peroxide is formed the cuprous ion causes a scission of the peroxide, and an oxidation of the cuprous to the cupric ion *(27)*. The cupric ion is then available for further reaction with the ascorbic acid.



The free radical formed by the scission of the peroxide may initiate further reaction chains, thus increasing the rate of fat oxidation.

Polyhydroxy compounds and their ability to chelate copper. It is clear from a study of molecular configuration (Table 2) that all of the substances, which are active antioxidants when in combination with ascorbic acid, possess at least two or more functional groups capable of chelation with copper so that one or more rings can be formed. There is also a clear indication that the presence of ascorbic acid improves the antioxidant quality of those compounds which form stable copper chelates. These compounds complex the copper so that it can not oxidize ascorbic acid and the latter is then capable of acting as a synergistic antioxidant.

It has been shown *(5)* that chelates formed from dihydroxy compounds are those of the α, β dihydroxy compounds to give five membered rings of the following type:

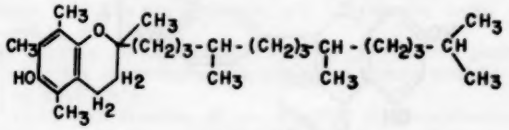


The properties used to demonstrate chelation, i.e., increased acidity and increased electrical conductance, indicated weak to no interaction when the hydroxyl groups were further apart. There would, therefore, be no chelation of copper, and thus no retardation of the prooxidant effect of ascorbic acid by substances of the meta and para type. An example of this would be the case of hydroquinone.

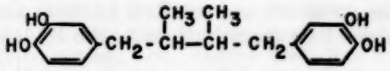
Ortho dihydroxy compounds such as dihydroxyphenylalanine, catechol, and gallates, while effective antioxidants, are apparently of no value as

TABLE 2

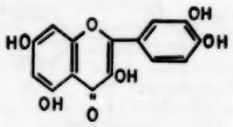
The structure of some polyhydroxy compounds



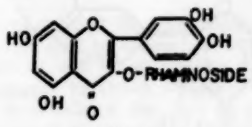
α-TOCOPHEROL



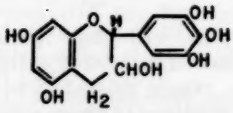
NORDIHYDROGUAIARETIC ACID



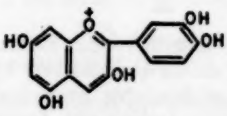
QUERCETIN



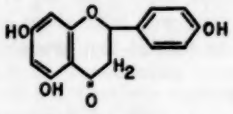
RUTIN



GALLOCATECHIN



CYANIDIN



NARINGENIN

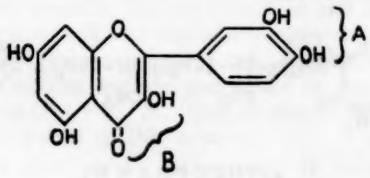
CATECHOL

inhibitors of the prooxidant effect of ascorbic acid possibly because they form soluble chelates which, although stabilized by resonance, are dissociable *(5)*.

Nordihydroguaiaretic acid, quercetin, myricitin, quercetrin, lutiolin, rutin, and gallocatechin are ideally suited as natural product sequestering agents since they are nontoxic, are structurally capable of copper complexing, and can form relatively insoluble copper complexes.

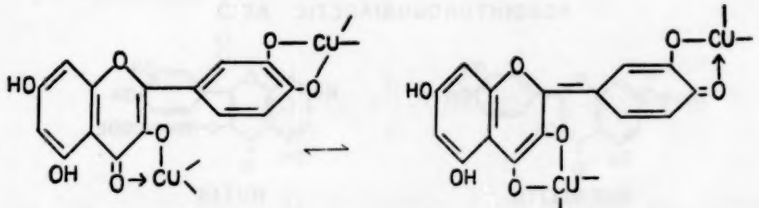
The greater activity of quercetin compared with rutin led Clark and Geissman *(8)* to propose that in the flavonoids the important complex form-

ing elements were the 3′, 4′ dihydroxy groupings, and the 3 hydroxy, 4 keto groupings shown as A and B in the formula for quercetin (I) below.



I

The importance of the structure was ascribed by these authors to the stabilization of the complex II by contributions to the resonance hybrid forms such as III.



II III

That chelation with copper actually takes place with these flavonoid compounds was shown qualitatively by the formation of colored substances (in solution or as insoluble precipitates) when cupric chloride was added to their alcoholic solutions *(8)*. Polarographic studies *(12)* have demonstrated that complexes are formed as shown by the disappearance or diminution of the normal copper wave, and the appearance of a new wave or a more negative half wave potential in mixtures containing copper sulfate and a number of flavonoid compounds. These agreed very well with those compounds which were found to be antioxidants, and to inhibit the prooxidant effect of ascorbic acid.

SUMMARY

A number of compounds were tested for their effect on rancidity alone and combined with ascorbic acid. Nordihydroguaiaretic acid and certain flavonoid compounds were effective antioxidants, and inhibited the prooxidant effect of ascorbic acid for prolonged periods of time.

A copper-ascorbic acid complex was believed to be the causative agent for the prooxidant effect of ascorbic acid in aqueous fat systems, and a hypothesis was proposed to account for the acceleration of fat rancidity by this complex. This hypothesis was supported by evidence that these compounds which were effective antioxidants in the presence of ascorbic acid were capable of forming stable copper chelates.

LITERATURE CITED

1. ABRAMSON, H. The oxidation of unsaturated fatty acids in normal and scorbutic guinea pigs. *J. Biol. Chem.*, **178**, 179 (1949).

2. BAILEY, A. E. *Industrial Oil and Fat Products.* 1951. Interscience Publishers, New York, N. Y.
3. BANKS, A. Method for studying the effect of antioxidants on the oxidation of aqueous suspensions of unsaturated fatty acids. *J. Soc. Chem. Ind.*, **63**, 8 (1944).
4. BAUERNFEIND, J. C. The use of ascorbic acid in processing foods. *Advances in Food Research*, **4**, 359 (1953).
5. BOESEKEN, J. Über die lagerung der hydroxy gruppen von polyoxy verbindungen im raum. Die configuration die gesättigen glykole und der α und β glykose. *Ber.* **46**, 2612 (1913).
6. CALKINS, V. P., AND MATTILL, H. A. Kinetics of the antioxygenic synergism of quinones with ascorbic acid in fat systems. *J. Am. Chem. Soc.*, **66**, 239 (1944).
7. CASSON, E. J. (Unpublished data. Florida State University, Tallahassee, Florida.)
8. CLARK, W. G., AND GEISSMAN, T. A. Potentiation of the effects of epinephrine by flavonoid (Vitamin P-like). *J. Pharm. Exptl. Therap.*, **95**, 363 (1949).
9. DULKIN, S. I., AND FRIEDEMANN, T. E. The role of dehydroascorbic and dehydroreductic acids in the browning reaction. *Food Research*, **21**, 519 (1956).
10. FEIGL, F. *Spot Tests.* 4th ed., 1954. Elsevier Press, New York, N. Y.
11. FLITMAN, R., AND FRIEDEN, E. (Unpublished data. Florida State University, Tallahassee, Florida.)
12. GEISSMAN, T. A. (Unpublished data. University of California at Los Angeles, Los Angeles, California.)
13. GOLUMBIC, C., AND MATTILL, H. A. Antioxidants and the autooxidation of fats XIII. The antioxygenic action of ascorbic acid in association with tocopherols, hydroquinones and related compounds. *J. Am. Chem. Soc.*, **63**, 1279 (1941).
14. HEULIN, E. E., AND STEPHENS, I. M. The copper catalyzed oxidation of ascorbic acid in fruit and vegetable suspensions. Australian *J. Sci. Res.*, **50**, 289 (1948).
15. LEHMANN, B. T., AND WATTS, B. M. Antioxidants in aqueous fat systems. *J. Am. Oil Chem. Soc.*, **28**, 475 (1951).
16. MARTELL, A. E., AND CALVIN, M. *Chemistry of the Metal Chelate Compounds.* 1952. Prentice Hall, Inc., New York, N. Y.
17. NAGY, J. J., BEADLE, B. W., AND KRAYBILL, H. R. Use of dried air in the active oxygen method of determining relative stabilities of fats. *Oil and Soap*, **22**, 123 (1945).
18. NORD, H. Kinetics and mechanism of the copper catalyzed autooxidation of ascorbic acid. *Acta. Chem. Scand.*, **9**, 442 (1955).
19. PENNEY, J. R., AND ZILVA, S. S. The chemical behavior of dehydro-l-ascorbic acid *in vitro* and *in vivo*. *Biochem. J.*, **37**, 403 (1943).
20. RICHARDSON, G. A., EL-RAFEY, M. S., AND LONG, M. L. Flavones and flavone derivatives as antioxidants. *J. Dairy Sci.*, **30**, 397 (1947).
21. SCARBOROUGH, D. A., AND WATTS, B. M. The prooxidant effect of ascorbic acid and cysteine in aqueous fat systems. *Food Technol.*, **3**, 152 (1949).
22. WATTS, B. M. Freeze curing of bacon. *Food Technol.*, **10**, 101 (1956).
23. WATTS, B. M., Oxidative rancidity and discoloration in meats. *Advances in Food Research*, **5**, 1 (1954).
24. WATTS, B. M., AND FAULKNER, M. B. Antioxidant effect of liquid smokes. *Food Technol.*, **8**, 158 (1954).
25. WATTS, B. M., AND LEHMANN, B. T. Ascorbic acid and meat color. *Food Technol.*, **7**, 194 (1952).
26. WATTS, B. M., AND WONG, R. Some factors affecting the antioxidant behavior of ascorbic acid with unsaturated fats. *Arch. Biochem.*, **30**, 110 (1951).
27. WEISSBERGER, A., AND LUVALLE, J. E. The autooxidation of ascorbic acid in the presence of copper. *J. Am. Chem. Soc.*, **66**, 700 (1944).

A METHOD FOR DETERMINING SIGNIFICANCE OF VOLATILE FLAVOR COMPOUNDS IN FOODS[a]

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(Manuscript received January 19, 1957)

One aspect of flavor research appears to be evolving rapidly as a science. This concerns identification of (volatile) flavor compounds and results from recently developed chromatographic and spectroanalytical tools. Such tools have enabled identification on a microscale heretofore impossible. It seems probable that comprehensive analyses of volatiles from many foods will soon find their way into the literature. When identification data are in hand, the question arises: which of the identified compounds are of flavor significance? Because of the relative nature of flavor and odor, answers to this question ordinarily have been presented as personal opinions. This state of affairs indicates the need for a more objective approach.

EXPERIMENTAL

In determining whether a particular compound is of significance in a flavor, 2 principal problems must be solved: (a) the concentration of the compound in the food product must be determined and (b) the flavor threshold-value for the compound must be revealed. The premise is that compounds which exceed threshold-level in a food are significant in its flavor, whereas those occurring below threshold are not. Although this premise may not hold in all instances, it does appear to have merit as a working principle. Concerning the problem of determining concentration of the compound in the food product, the answer frequently is inherent in the identification work. If not, it usually can be secured by one of the many sensitive analytical methods. The second problem, that of determining threshold-values for flavor compounds, need not be overly difficult. Threshold-studies may be very precise, elaborate and time-consuming. However, a fairly simple version, which appears adequate for the purpose, has been employed at this laboratory. This procedure is as follows:

A series of samples are prepared containing the compound in a range from zero concentration to well above the anticipated threshold-area. Samples can be made by using water or any other convenient and appropriate medium. The samples are adjusted to approximately room temperature and, using a dentist's chair type of environment, are presented by spray technique to the taste observers. In this technique, a 3-ml. sample is drawn into a 5-ml. graduated pipette by means of an attached rubber bulb. The sample is then sprayed into the mouth of the taste observer who, after evaluation of the sample, merely indicates his judgment as positive or negative concerning presence of the compound. One or two preliminary presentations of the zero and highest concentration samples are made to each observer so that he will be properly oriented to make judgments. Samples are then presented in random order. Rinsing the mouth and rest periods between observations are left to the discretion of the individual observer. Five observers, recruited from available personnel, are used. At times individuals showing extremes of sensitivity or insensitivity to the compound under study will be encountered. These should be replaced by individuals who conform more nearly to the norm of the group. Five presentations per concentration per observer generally afford adequate data. This yields a total of 25 judgments for a given concentration when 5 observers are employed. Data

[a] Authorized for publication on January 10, 1957 as paper No. 2118 in the journal series of the Pennsylvania Agricultural Experiment Station.

may be plotted as shown in Figure 1, with the 50% level of positive responses being selected as the average threshold for the compound.

RESULTS AND DISCUSSION

Flavor threshold-data secured by this method for a number of compounds are presented in Table 1. The extremely low threshold-values for the sulfur compounds in this table are notable.

TABLE 1

Flavor thresholds for some volatile compounds

Compound	Medium of evaluation	Threshold [1] (p.p.m.)	Flavor quality
Butyric acid	Homog. milk	25	rancid milk
Caproic acid	Homog. milk	14	rancid milk
Acetaldehyde	Skim milk	1.3	burnt, malty
Acetone	Skim milk	500 [2]	chemical, medicinal
Butanone	Skim milk	50	fermented, sour milk
Methional	Skim milk	0.05	broth-like
Methyl disulfide	Skim milk	0.021	putrid, burnt
Methyl sulfide	Water	0.012	malty, cowy
Methyl mercaptan	Water	0.002	putrid, cooked cabbage

[1] Average values derived from results of five observers.
[2] Individual observers varied widely in their sensitivity to acetone. Thresholds ranged from 40 to 1000 p.p.m.

It will be clear that this procedure for determining flavor thresholds is not meant to be inflexible. Psychometric experimentation ordinarily involves many variables. The manner in which these are controlled will be determined best by the specific requirements of a problem.

Unfortunately, there are unanswered questions in the field of flavor perception which may have a bearing on the proposed method of evaluating flavor significance. One consideration of importance concerns the role of compounds present at sub-threshold-levels in the flavor of foods. Another involves the possible masking effect of a food system on flavor substances present at levels above threshold. Despite these finer points of flavor science, it is felt that the proposed practical method may provide guiding information along the lines needed.

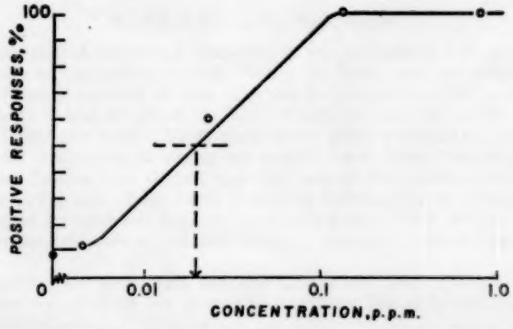

Fig. 1. Average threshold-data for methyl disulfide in skim milk using five taste observers. (Average threshold-value at the 50% level of positive response—0.021 p.p.m.).

SUMMARY

A method is presented for evaluating the significance of volatile compounds in the flavor of foods. This method is based on determining concentration of the compound in a food and deriving its flavor threshold value psychometrically. Compounds present at levels in excess of their threshold-range are considered to make a direct contribution to flavor, whereas those below threshold are thought to exert little or no effect. A relatively simple procedure for determining flavor thresholds is described and threshold data for a number of compounds, including some containing reduced sulfur, are presented.

EFFECT OF MALEIC HYDRAZIDE ON THE COMPOSITION OF RADISHES (RAPHANUS SATIVUS)[a]

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(Manuscript received January 28, 1957)

Maleic hydrazide, 1,2-dihydro-3,6-pyridazinedione, is generally regarded as an inhibitor of plant growth. Previous studies *(2, 4, 7, 8)* have indicated that the shelf life of many economically important root crops and other storage organs (tubers) can be extended by proper treatment of plants with maleic hydrazide. The visual effect of treatment is the inhibition of growth in the storage organ, which suggests that the nutrient value of the storage organ is preserved. Growth in the storage product may be effectively inhibited and its storage life prolonged by preharvest foliar sprays of maleic hydrazide at the proper stage of plant development. This method of treatment, although not the only effective one, is particularly desirable since it circumvents partially the problem of large amounts of residue remaining in the product used for food. Since application is not directly on the root or tuber, residues which remain must have been absorbed by and from the foliage and translocated by the plant to the storage organ.

When sprayed on the radish foliage prior to harvest, maleic hydrazide is effective in preventing new shoot and secondary root growth of topped and packaged radishes subsequently held in storage until marketed *(1)*. Since commercially topped radishes are frequently topped mechanically by clipping near the base of the petioles without removal of the vegetative growing points, subsequent growth during storage is of some concern. The inhibition of new shoot and root growth is important for preserving the nutrient value of the storage organ and for the product to meet existing market standards *(3)*.

The effect of a preharvest foliar application of maleic hydrazide on the gross composition and mineral composition of the radish is reported herein.

MATERIALS AND METHODS

Radishes were seeded in ground bed plots in the greenhouse and grown under conditions comparable to commercial culture. When the fleshy root had obtained a marketable size, the foliage of one-half of each plot was sprayed with a 2500 p.p.m. solution of maleic hydrazide at a rate of about one liter of solution per 100 sq. ft. (equivalent to *ca* 6 pounds MH-40[b] per acre). Forty-eight hours after treatment 3 replicate weight equivalent samples containing 25 plants per sample were taken from each of 3 control and treated plots. Leaves were clipped near the base of the petioles without removal of the vegetative growing tips. Leaf tissue was dried immediately in a forced air oven at 60° C. and prepared for chemical analysis by grinding in a Wiley Mill to pass a 60-mesh sieve. Topped roots were then stored 168 hours in ventilated polyethylene containers at

[a] Journal Article No. 1967 from the Michigan Experiment Station, East Lansing. This research was supported by the Horace H. Rackman Research Endowment of Michigan State University.

[b] MH-40 is a sodium salt formulation containing 40 percent maleic hydrazide obtained from Naugatuck Chemical, Naugatuck, Conn.

the prevailing greenhouse temperatures (55 to 70° F.) until new shoot and secondary root growth was apparent on the control samples. The leaf petiole bases and new remaining shoot growth, if present, were then removed, the fleshy roots dried, and ground for chemical analysis. A second sampling was conducted as described previously except that radishes were harvested 216 hours after treatment with maleic hydrazide. Both the leaf and root tissue were prepared for chemical analysis without storage. This harvest corresponded to the time elapse of 48 hours after treatment plus the 168 hours of storage for the previous samples.

Carbohydrates were determined by a modification of methods described by Sell *et al. (5)*. A semi-micro Kjeldahl procedure using potassium sulfate and copper sulfate as catalyst was used for the nitrogen determination. Mineral analyses were determined spectrographically with the exception of sodium and potassium which were evaluated flame photometrically.

RESULTS AND DISCUSSION

Radishes treated with maleic hydrazide to control growth in storage, and thereby to extend the shelf life, raises the question of the effect of chemical treatment on the nutritional quality of this food product. Data in Table 1 indicate that radishes (fleshy roots) harvested 48 hours after maleic

TABLE 1

The effect of maleic hydrazide[1] on the gross composition of radishes (Results expressed as percent dry weight)[2]

Plant Constituent	Fleshy Roots				Leaves			
	After 48 hrs. plus 168 hrs. storage		After 216 hours no storage		After 48 hours no storage		After 216 hours no storage	
	Control	MH-Treated	Control	MH-Treated	Control	MH-Treated	Control	MH-Treated
Dry weight	4.45	4.57	5.42	6.58**	6.54	6.68*	6.43	9.90**
Ether extract	1.82	2.15**	2.37	1.20**	4.02	3.85	3.68	3.70
Kjeldahl nitrogen	3.32	3.25	2.82	2.59*	5.82	5.59*	5.42	4.71**
Reducing sugars	19.41	19.93	29.76	28.96	0.67	0.44	2.75	5.62**
Non-reducing sugars	1.80	2.36*	0.43	2.63**	3.07	3.33	0.00	0.58**
Starch	0.62	0.38**	0.25	0.27	0.76	1.45**	0.25	0.35
Polysaccharides[3]	1.72	1.54	3.76	3.00	2.67	2.56	3.19	2.90
Total carbohydrates determined	23.55	24.21	34.17	35.47	7.14	7.77	6.19	9.45**

[1] Applied at a concentration of 2500 p.p.m. as a preharvest foliar spray.
[2] All results are the mean values obtained from single determinations on three replicate samples.
[3] Acid hydrolyzable polysaccharides other than starch.
* Difference between treated and control significant (P = 0.05).
** Difference between treated and control highly significant (P = 0.01).

hydrazide treatment and then stored as topped radishes for 168 hours remained at essentially the same nutritional level as reflected by the gross composition analyses. This was true even though new shoot and secondary root growth was common on the control radishes while none occurred on the treated lots after 168 hours' storage. Results of the chemical analyses (Table 1) show statistically significant increases in ether extract and non-reducing sugar and decreases in starch. Although these differences were significant, they do not reflect a large change in composition or nutritional quality.

Even though a 48-hour treatment with maleic hydrazide effectively prolonged storage quality without essentially altering the composition, further investigations of the effects of maleic hydrazide on the composition

was also of interest. Data in Table 1 shows that prolonged treatment with maleic hydrazide (216 hours) resulted in more pronounced changes in composition. This treatment increased the dry weight by about 1% and the non-reducing sugar by 2%. A slight decrease in reducing sugars and a decrease in the amount of ether extractives was observed; the other components remained essentially unchanged.

Although the composition of the leaf tissues of maleic hydrazide treated radishes is of little nutritional interest since these parts are not usually consumed, it seemed appropriate for the sake of completeness to determine leaf composition. Results of these analyses (Table 1) indicate that 48 hours after treatment the only significant differences in composition were increased dry weights and approximately twice the quantity of starch when compared with the corresponding control tissue. In addition, the nitrogen content was decreased slightly. Like the fleshy root, greater changes in composition occurred 216 hours after treatment. Significant increases in dry weight, reducing and non-reducing sugars were observed in the treated leaf samples, while the nitrogen content decreased.

The water relationship in both the treated roots and leaves was altered by application of maleic hydrazide. Most marked effect occurred in the latter after the longer exposure to the chemical. The increase in reducing and/or non-reducing sugars suggests an inhibition of respiration *(6)*.

Since radishes are a good source of minerals for the human diet, it was of interest to determine the effect of chemical treatment on the mineral level. The mineral content of the roots and leaves is presented in Table 2. These data indicate that the chemical treatments had little effect on the mineral composition. The only significant changes as noted in Table 2 were increases in sodium in the leaves 48 hours after treatment and decreases in calcium and phosphorus 216 hours after treatment.

TABLE 2

The effect of maleic hydrazide[1] on the mineral composition of radishes (Results expressed as percent dry weight)[2,3]

Mineral	Fleshy Roots				Leaves			
	After 48 hrs. plus 168 hrs. storage		After 216 hours no storage		After 48 hours no storage		After 216 hours no storage	
	Control	MH-Treated[1]	Control	MH-Treated	Control	MH-Treated	Control	MH-Treated
B	0.0039	0.0046	0.0025	0.0024	0.0038	0.0037	0.0023	0.0023
Ca	0.35	0.34	0.78	0.68	1.50	1.39	2.01	1.66*
Cu	0.0013	0.0018	0.0012	0.0035	0.0023	0.0064	0.0010	0.0013
Fe	0.009	0.0016	0.016	0.018	0.028	0.026	0.018	0.017
K	8.66	8.32	6.51	6.13	6.58	6.31	7.69	7.20
Mg	0.29	0.30	0.21	0.17	0.57	0.54	0.41	0.39
Mn	0.0024	0.0027	0.0023	0.0021	0.0087	0.0088	0.0084	0.0081
Na	0.257	0.309	0.175	0.210	0.403	0.529**	0.408	0.472
P	0.61	0.75	1.10	1.09	0.35	0.31	0.44	0.30**
Zn	0.0023	0.0022	0.0017	0.0022	0.0050	0.0044	0.0047	0.0041

[1] Applied at a concentration of 2500 p.p.m. as a preharvest foliar spray.
[2] All results are the mean values obtained from duplicate determinations on two replicate samples.
[3] All determinations were obtained spectrographically except Na and K, which were evaluated flame photometrically.
* Difference between treated and control significant ($P = 0.05$).
** Difference between treated and control highly significant ($P = 0.01$).

SUMMARY

A preharvest foliar spray of a 2500 p.p.m. solution of maleic hydrazide is effective in prolonging the storage, or shelf life, of topped radishes by retarding new shoot and root growth. Gross composition and mineral analyses 48 hours after treatment plus 168 hours of storage as topped radishes indicate that the nutrient value of the fleshy roots remained essentially unchanged. However, 216 hours after treatment there are greater changes in gross composition in the fleshy radish root, and in both mineral and gross composition of the leaves. The greatest differences in composition resulting from maleic hydrazide treatment were increases in dry weight, non-reducing sugars, and a reduction in Kjeldahl nitrogen.

Acknowledgment

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LITERATURE CITED

1. Dewey, D. H. and Wittwer, S. H. Chemical control of top growth of pre-packaged radishes. *Proc. Am. Soc. Hort. Sci.*, **66**, 322-330 (1955).
2. Howat, M. G. The effects of maleic hydrazide on plant development and root quality of *Raphanus sativus* L. (M. Sc. thesis, University of Alberta, Edmonton, 1953).
3. Lennartson, R. W. United States standards for radishes. U. S. Dept. Agr. Mkt. Service, 1954.
4. Patterson, D. R., Wittwer, S. H., Weller, L. E., and Sell, H. M. The effect of preharvest foliar sprays of maleic hydrazide on sprout inhibition and storage quality of potatoes. *Plant Physiol.*, **27**, 135-142 (1952).
5. Sell, H. M., Johnston, F. A. and Lagasse, F. S. Changes in the chemical composition of the tung fruit and its component parts. *J. Agr. Res.*, **73**, 319-334 (1946).
6. Weller, L. E., Ball, C. D., Wittwer, S. H. and Sell, H. M. Comparative effects of maleic hydrazide on the DPNH oxidase system of *Raphanus sativus*. (In preparation.)
7. Wittwer, S. H., Sharma, R. C., Weller, L. E. and Sell, H. M. The effect of preharvest foliage sprays of certain growth regulators on sprout inhibition and storage quality of carrots and onions. *Plant Physiol.*, **25**, 539-549 (1950).
8. Wittwer, S. H. and Patterson, D. R. Inhibition of sprouting and reduction of storage losses in onions, potatoes, sugar beets and vegetable root crops by spraying plants in the field with maleic hydrazide. Mich. Agr. Expt. Sta. *Quart. Bull.*, **34**, 3-8 (1951).

COMPARISON OF XANTHOPHYLLS FROM LEAVES AND ORANGE JUICE

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(Manuscript received February 2, 1957)

Previous work at this Laboratory using countercurrent distribution and chromatography has demonstrated the complexity of the carotenoid fraction of Valencia orange juice *(3, 4, 5)*. In addition to 10 carotenoids not containing cyclic ether groups (such as α- and β-carotenes, cryptoxanthin, and lutein), 5 carotenoid 5,6-epoxides are usually found, together with the isomeric 5,8-epoxides.[a] The 5,8-epoxides are formed by the action of acid on the 5,6-epoxides, and may be in part artifacts. Three of the 5,6-epoxides appear to be polyols of unknown structure. The question has been raised whether some of these substances, in particular the polyols, might be artifacts arising from other carotenoids such as violaxanthin in the method of isolation used. This included extraction of pigment with acetone, saponification, separation into fractions by countercurrent distribution in a Craig apparatus, and finally chromatography of these fractions.

Strain *(11)* demonstrated that the carotenoid mixture found in leaves of various species was remarkably uniform; the mixture was considerably simpler in composition than the carotenoid mixture isolated by the present workers from orange juice. Accordingly, the carotenoids of leaves were investigated by essentially the same methods as were used in the investigation of the carotenoids of orange juice. The pigment mixture obtained from leaves corresponded closely to that found by Strain. The diol xanthophylls obtained from leaves had all been obtained previously from orange juice, though differing considerably in relative proportions. Violaxanthin, a rather labile carotenoid, was a major component of both. Lutein 5,6-epoxide-like and flavoxanthin-like (lutein 5,8-epoxide) components have been found recently as very minor constituents in Florida Valencia orange juice. The carotenoid polyol fraction isolated from leaves was much simpler in composition than the corresponding fraction obtained from orange juice; the two polyol fractions had no components in common. This indicates that the polyols are natural (actual) constituents of orange juice and of leaves, and are not artifacts.

The violaxanthin-like constituent, which is a major component of leaf xanthophylls, was shown in the present work to be violaxanthin (zeaxanthin 5,6,5′,6′-diepoxide) in confirmation of the work of Strain *et al. (9, 10, 12)* and not the spectroscopically indistinguishable lutein 5,6-epoxide as claimed by Karrer *et al. (7)*. A very minor fraction, which appeared to be lutein 5,6-epoxide, was isolated from leaves in the present work.

In agreement with the work of Strain *(11)*, zeaxanthin and neoxanthin were found in leaves. These pigments had been found in corn seedlings

[a] In earlier publications by the present authors *(3, 4, 5)*, the 5,6- and 5,8-epoxides were referred to as epoxides and furanoxides, respectively.

by Moster *et al. (8)* and in alfalfa by Bickoff *et al. (1)*. Neither of these pigments was found in leaves by Karrer *et al. (7)*. Evidence was obtained in the present work that neoxanthin is a trihydroxy-5,6-epoxide occurring in a mono-cis form not accompanied by the corresponding all-trans isomer. Neoxanthin and its 5,8-epoxide isomers were the only polyols obtained from leaves.

EXPERIMENTAL

This run was carried out on 100 leaves (71 g.) obtained from suckers on Dutch elm trees (*Ulmus hollandica* Mill.) in mid-August.

Extraction of leaf pigments. Elm leaves were allowed to stand overnight at 7° C. in acetone-petroleum ether (3:7 by volume); calcium carbonate was added and the mixture was then blended and filtered on a sintered-glass funnel, and washed with the same solvent mixture. The solid residue was similarly extracted twice more, then 3 times with acetone alone. The combined extracts were evaporated *in vacuo*, the aqueous residue extracted with ether, the ethereal extracts evaporated *in vacuo*, and the residue saponified as previously described for orange juice lipides *(3)*. The saponified carotenoid fraction (the unsaponifiable fraction in fat saponification) was dissolved in a mixture of petroleum ether and 90% methanol the lower layer was separated and extracted 6 times with petroleum ether. The 90% methanol solution was evaporated *in vacuo*, the aqueous residue extracted with ether, the ethereal extracts evaporated *in vacuo* and the residue dissolved in the upper layer of the solvent system used in countercurrent distribution (see below).

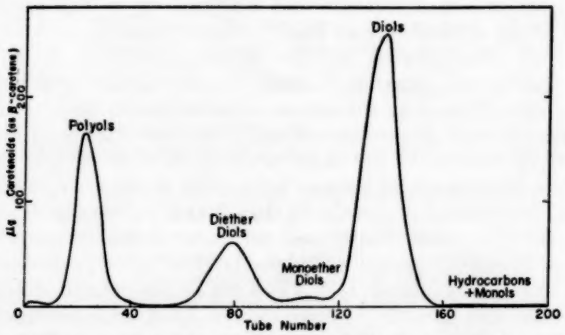
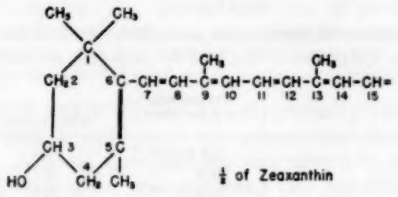


Figure 1. Countercurrent distribution of hypophasic saponified carotenoids from elm leaves. System: Benzene, petroleum ether, and 87% methanol.

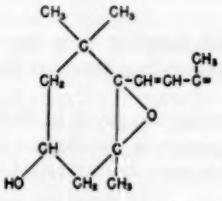
Countercurrent distribution. Runs were carried out as in earlier work *(3)* in a 200-tube Craig apparatus with the solvent system consisting of petroleum ether, benzene, and 87% methanol (1 to 1 to 1.15 by volume). Color of the contents of alternate tubes of the Craig apparatus was measured as previously described *(3)*; solutions were then combined into 4 fractions which were chromatographed essentially as previously described *(3)* on columns of magnesia (Westvaco No. 2642) plus diatomaceous earth (1 to 1 by volume). Color bands were fractionally eluted by solutions of ethanol in petroleum ether or benzene, and spectral absorption curves were run in a Cary recording spectrophotometer.

Iodine treatment. Two ml. of a carotenoid solution in benzene having an adsorbance of around 1.5, measured in a 1-cm. cell in a Cary recording spectrophotometer, was treated with 0.2 ml. of a 0.001% solution of iodine in benzene and then exposed to light from a daylight fluorescent lamp at approximately 260 foot candles for 30 minutes. Spectral absorption curves were then run within a few minutes.

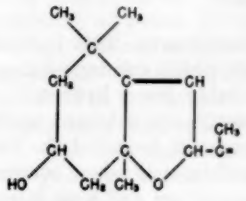
Hydrochloric acid-ether test and treatment with hydrochloric acid in methanol. These were carried out as previously described *(5)*.



½ of Zeaxanthin



Violaxanthin



Auroxanthin

Figure 2. Partial structural formulas for zeaxanthin, violaxanthin (zeaxanthin 5,6,5′,6′-diepoxide) and auroxanthin (zeaxanthin 5, 8, 5′,8′-diepoxide).

RESULTS AND DISCUSSION

Countercurrent distribution of saponified elm leaf carotenoids. Although leaf xanthophylls occur almost entirely in a non-esterified state, saponification was found to be necessary to remove the chlorophylls and their decomposition products which obscured the carotenoids during countercurrent distribution and subsequent color measurement. Further, countercurrent distribution runs on unsaponified material with the solvent system used here were impractical because of extreme emulsion formation. In order to prevent the slowly separating emulsions encountered in a preliminary run, the saponified carotenoid fraction was partitioned between petroleum ether and 90% methanol. Petroleum ether extraction removed most of the emulsion formers, probably sterols and wax alcohols. Chromatography of the petroleum ether extract showed that β-carotene had been extracted into it quantitatively, as well as a part of the lutein; small amounts of other pigments were present.

On countercurrent distribution of the 90% methanol-soluble material no difficulty with emulsions was encountered. Distribution of the pigments, measured with a 440 mμ filter and calculated as β-carotene *(3)*, is given in Figure 1. The hydrocarbon and monol fraction, which would be expected to occur at about tube 190, was practically absent. Three well-defined peaks were present, while the monoether diol fraction (IIIB), which was a major fraction of orange juice carotenoids *(3)*, was quite small. The percentages of the several fractions together with the N_{100} values are given in Table 1; N_{100} values previously found for the corresponding fractions from orange juice *(3)* are included. The N_{100} values of all of the fractions were in good agreement except for a somewhat higher value for the polyol

TABLE 1

Countercurrent distribution of hypophasic saponified carotenoids from elm leaves (System: Petroleum ether, benzene, and 87% methanol)

Fraction	Percentage (as β-Carotene)	N_{100}[1]	
		Elm Leaves	Orange Juice
Hydrocarbons + monols	0.1 (48.9)[2]	—	96
Diols	29.6	70	70
Monoether diols	0.9	55	56
Diether diols	7.8	40	39
Polyols	12.6	12	9

[1] N_{100} = tube number of maximum calculated per 100 transfers.
[2] Pigment extracted by partition between petroleum ether and 90% methanol; included some lutein and other diols.

fraction from leaves. This indicates that this fraction from leaves consists of less hydrophilic substances than the corresponding fraction from orange juice (probably fewer hydroxyl groups).

The constituents obtained on chromatography are given in Table 2. Non-ether carotenoids included the two major pigments β-carotene and lutein, also zeaxanthin, and minor components which were tentatively identified as hydroxy-α-carotene and a cis-lutein.

Monoether diols. Chromatography of this fraction yielded 4 minor bands which were tentatively identified as lutein 5,6-epoxide, antheraxanthin, flavoxanthin (lutein 5,8-epoxide), and mutatoxanthin. After treatment with hydrochloric acid in methanol, the absorption maxima of the flavoxanthin-like substance were practically unchanged. This eliminates the possibility that this component might be a luteoxanthin (zeaxanthin 5,6,5′,8′-diepoxide) *(5)*. It was pointed out earlier *(5)* that Strain's "isolutein" *(11)* may be the same as antheraxanthin. Antheraxanthin (zeaxanthin 5,6-epoxide) and the mutatoxanthins (zeaxanthin 5,8-epoxides) were found to be major pigments of orange juice *(5)*.

Diether diols. Chromatography yielded 10 bands (Table 2), all of which were similar to bands previously obtained from the corresponding fraction of orange-juice carotenoids. These bands probably all originated from violaxanthin by *in situ* isomerization, either trans-cis or 5,6-epoxide-5,8-epoxide. On treatment with iodine in light the spectral absorption maxima in benzene of the two violaxanthin fractions changed to 481, 450, 425, and 482, 451, 426 mμ, respectively, indicating them to be trans-cis isomers. All-trans violaxanthin fractions from elm leaves and orange juice showed no separation in a mixed chromatogram; neither did the two cis-violaxanthins from the same sources, indicating identity of the two pairs. The violaxanthin-like pigment from orange juice was shown to be violaxanthin and not lutein 5,6-epoxide by the products of the reaction with hydrochloric acid *(5)*; the spectral absorption maxima of the principal product corresponded to those of auroxanthin and not flavoxanthin. This method of distinguishing between lutein 5,6-epoxide and violaxanthin was described by Karrer *et al. (7,6,* p. 206*)*. This supports the identification of the violaxanthin-like pigment from leaves as violaxanthin (zeaxanthin 5,6,5′,6′-diepoxide) as reported by Strain *(9, 10, 12)* and not as lutein 5,6-epoxide, which has a very similar spectral absorption curve. Karrer *et al. (7)* reported that lutein 5,6-epoxide is a major leaf pigment.

As postulated earlier *(5)*, cis-violaxanthin is probably identical with the violeoxanthin of Strain *et al. (12)*. Violeoxanthin-like fractions were obtained from corn seedlings by Moster *et al. (8)* and from alfalfa by Bickoff *et al. (1)*.

Luteoxanthins *a* and *b* (Table 2) are probably identical with Strain's flavoxanthins *c* and *b (11)* and are apparently zeaxanthin 5,6,5′,8′-di-epoxide isomers. Because flavoxanthin was shown to be lutein 5,8-epoxide *(6)*, the name luteoxanthin was proposed *(5)* for these substances which have spectral absorption curves very similar to that of flavoxanthin. Strain *(11,* p. 66*)* obtained a fraction from leaves which appears to have been auroxanthin (absorption maxima in carbon disulfide, 455 and 426 mμ). In the present work 4 auroxanthin-like bands were obtained. One appeared to be a cis-isomer while the others were apparently all-trans. It was pointed out earlier *(5)* that 5,8-epoxides are sometimes obtained as pairs of bands with practically identical spectral absorption curves. Since auroxanthin is 5,8,5′,8′-di-epoxide, 3 such bands were obtained in this case.

Polyols. Two pairs of bands were obtained, each with practically identical spectral absorption maxima (Table 2). The first pair was eluted by 7% ethanol in petroleum ether, whereas the trollixanthin-like polyol from orange juice required 15% ethanol in petroleum ether for ready elution. The lower member (on the column) of this pair of bands was considerably greater than the upper. The spectral absorption maxima of these fractions agreed closely with those reported by Strain *(11)* for neoxanthin; the spectral absorption curves were very similar to that of cis-violaxanthin. The lower of these bands was one of the major xanthophylls of leaves and was evidently neoxanthin, which Strain found to be a universal major constituent of leaf xanthophylls. The upper band appeared to be a closely related substance; hence the two are referred to here as neoxanthins *a* (the major band) and *b*.

After treatment with iodine in light the spectral absorption maxima in benzene of neoxanthins *a* and *b* became 480,450,425 and 481, 450, 425 mμ, respectively (increases of 2 to 3 mμ). This shows the presence of a cis-bond; none of the corresponding all-trans isomers were found accompanying neoxanthins *a* and *b*. The neoxanthins apparently have the same conjugated double-bond system as violaxanthin. Both neoxanthins gave a pale blue color in the hydrochloric acid-ether test, indicating the probable presence of one cyclic ether group. Strain *(11)* reported that neoxanthin gave a deep blue color in this test, but later Strain *et al. (12)* stated that neoxanthin did not give a blue color.

The other two bands in the polyol fraction had almost identical spectral absorption curves, with maxima 16 to 18 mμ lower in wave length than the neoxanthins; the curves resembled closely those of flavoxanthin, luteoxanthins, and the trollichrome-like fraction previously obtained from orange juice *(5)*. These bands were eluted from the column considerably more readily than the last of the 3 substances named above and the behavior on countercurrent distribution was different from that of the first two. It appears that these bands are 5,8-epoxide isomers which were formed from neoxanthin *in situ*. When neoxanthin *a* was treated with hydrochloric acid in methanol, two bands apparently the same as the 5,8-epoxide isomers above were obtained, confirming neoxanthin *a* as a 5,6-epoxide. The action

TABLE 2

Carotenoid constituents obtained from elm leaves

Component	Solvent[1]	Spectral Absorption Maxima, mμ[2]	Eluent[1]	%[3]
Hydrocarbons				
β-carotene	PE	476, 450, (426), 335	B	29
Monols				
"Hydroxy-α-carotene"	PE	473, 445, (422)	1% E/PE	0.5
Diols				
Lutein	B	486, 456, 434, 337	3.5% E/PE	41
Cis-lutein?	B	483, 454, (430), 338	5% E/PE	2.4
Zeaxanthin	B	490, 462, (436), 342	5% E/PE	0.9
Monoether Diols				
Lutein 5,6-epoxide	B	483, 451, 426	3.5% E/PE	0.1
Antheraxanthin	B	484, 455, (428)	5% E/PE	0.1
Flavoxanthin	B	460, 432, 407	7% E/PE	0.2
Mutatoxanthin	B	463, 436, (412)	15% E/PE	0.2
Diether Diols				
Violaxanthin	B	483, 453, 427, 337, (322)	5% E/PE	2.0
Cis-violaxanthin	B	477, 447, 423, 336, 323	7% E/PE	1.1
Luteoxanthin *a*	B	460, 432, 407, 319, 308	7% E/PE	1.5
Luteoxanthin *b*	B	460, 432, 407, 320, 309	10% E/PE	2.2
Cis-luteoxanthin *a*	B	456, 429, 406, 318, 306	15% E/PE	0.5
Cis-luteoxanthin *b*	B	455, 427, 406, 319, 306	25% E/PE	0.4
Cis-auroxanthin	B	432, 407, 385, 303	20% E/B	0.2
Auroxanthin *a*	B	436, 411, 388, 301	20% E/B	1.0
Auroxanthin *b*	B	436, 410, 388, 303	20% E/B	1.6
Auroxanthin *c*	B	437, 411, 389, 302	20% E/B	0.7
Polyols				
Neoxanthin *a*	B	478, 448, 424, 336, (323)	7% E/PE	6.7
Neoxanthin *b*	B	478, 448, 423, 336, 323	10% E/PE	1.4
Neoneoxanthin *a*	B	460, 432, 408, 319, 306	15% E/PE	2.0
Neoneoxanthin *b*	B	460, 432, 407, 319, 306	25% E/PE	2.1

[1] PE = petroleum ether; B = benzene; E = ethanol.
[2] Values in parentheses are approximations for humps or shelves on spectral absorption curves.
[3] Based on adsorbancies at principal maxima.

of hydrochloric acid in methanol on neoxanthin and its 5,8-epoxide isomers will be reported in more detail later. These two 5,8-epoxide bands resemble two substances obtained by Strain *et al. (12)* by heating neoxanthin in *n*-propanol for several hours at 100° C.; these substances were named neoneoxanthins *A* and *B*.

Strain *(11)* obtained analyses of neoxanthin which indicated the formula to be $C_{40}H_{56}O_4$. One of the oxygen atoms is a 5,6-epoxide. The behavior on countercurrent distribution indicates the presence of more than 2 hydroxyl groups; hence neoxanthin is probably a triol 5,6-monoepoxide.

Cholnoky *et al. (2)* recently examined the carotenoids of the leaves of the paprika plant and reported the presence in the hypophasic fraction of three major pigments—lutein (xanthophyll), violaxanthin, and "foliaxanthin"—together with much smaller amounts of antheraxanthin and "foliachrome." "Foliaxanthin" is apparently the same as neoxanthin. The 5,6-epoxide character of this pigment was recognized; the corresponding 5,8-epoxide was named "foliachrome."

SUMMARY

The xanthophylls of elm leaves and orange juice were compared. The diols obtained from leaves had all been found previously in orange juice carotenoids but in quite different proportions. The polyols from leaves were different from those of orange juice. In leaves lutein was the principal xanthophyll and was accompanied by much smaller amounts of zeaxanthin. The second most abundant xanthophyll in leaves was violaxanthin; its identity was confirmed as zeaxanthin 5,6,5′,6′-diepoxide and not as lutein 5,6-epoxide. Very minor amounts of pigments tentatively identified as lutein 5,6-epoxide and antheraxanthin were found. Neoxanthin, a major xanthophyll in leaves, was different from the trollixanthin-like polyol of oranges. It is apparently a trihydroxy 5,6-epoxide with one cis-bond and is unusual in that the corresponding all-trans isomer does not occur with it.

LITERATURE CITED

1. Bickhoff, E. M., Livingston, A. L., Bailey, G. F., and Thompson, C. R. Xanthophylls in fresh and dehydrated alfalfa. *J. Agr. Food Chem.*, **2**, 563 (1954).
2. Cholnoky, L., Györgyfy, K., Nagy, E., and Pánczél, M. Untersuchungen über Carotinoid-Farbstoffe. I. Die Farbstoffe des roten Tomatenförmigen Paprikas. *Acta chim. hung.*, **6**, 143 (1955).
3. Curl, A. L. Application of countercurrent distribution to Valencia orange juice carotenoids. *J. Agr. Food Chem.*, **1**, 456 (1953).
4. Curl, A. L., and Bailey, G. F. The state of combination of the carotenoids of Valencia orange juice. *Food Research*, **20**, 371 (1955).
5. Curl, A. L., and Bailey, G. F. Polyoxygen carotenoids of Valencia orange juice. *J. Agr. Food Chem.*, **2**, 685 (1954).
6. Karrer, P., and Jucker, E. *Carotenoids.* 1950. Elsevier Publishing Company, New York, N. Y.
7. Karrer, P., Krause-Voith, E., and Steinlin, K. Ein neuer Blattfarbstoff: Xanthophyllepoxyd. *Helv. Chim. Acta*, **31**, 113 (1948).
8. Moster, J. B., Quackenbush, F. W., and Porter, J. B. The carotenoids of corn seedlings. *Arch. Biochem. Biophys.*, **38**, 287 (1952).
9. Strain, H. H. Leaf xanthophylls: The action of acids on violaxanthin, violeoxanthin, taraxanthin and tareoxanthin. *Arch. Biochem. Biophys.*, **48**, 458 (1954).
10. Strain, H. H. Leaf xanthophylls. *J. Am. Chem. Soc.*, **70**, 1672 (1948).
11. Strain, H. H. Leaf xanthophylls. Carnegie Institution, Publication 490, Washington, D. C. (1938).
12. Strain, H. H., Manning, W. M., and Hardin, G. Xanthophylls and carotenes of diatoms, brown algae, dinoflagellates, and sea-anemones. Biol. Bull., **86**, 169 (1944).